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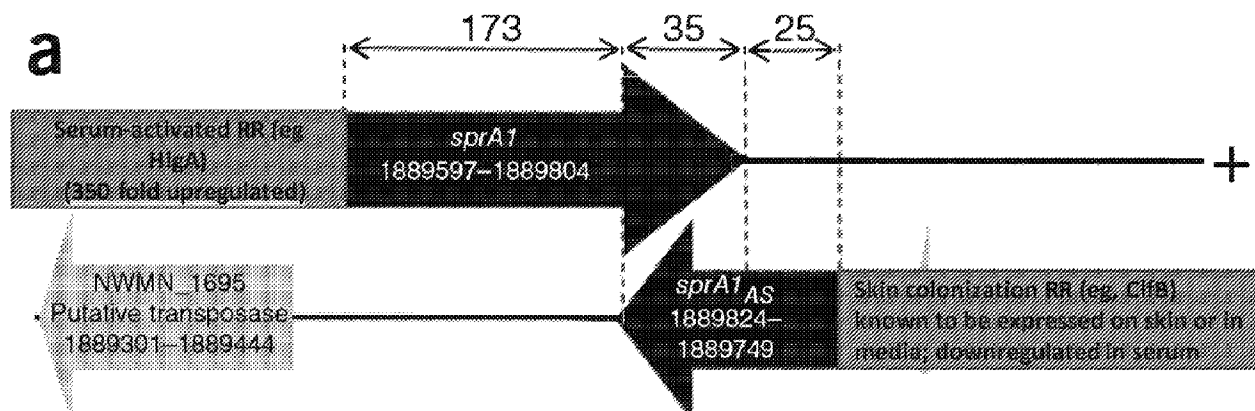


FIG 1

(57) **Abrégé/Abstract:**

Methods and compositions are provided for durably influencing microbiological ecosystems (microbiomes) in a subject in order to prevent infection and reduce recurrence of infection by microorganisms. In some embodiments, compositions and methods are provided for the creation and use of molecularly-modified bacterial strains with the potential to prevent a variety of microorganism infections.

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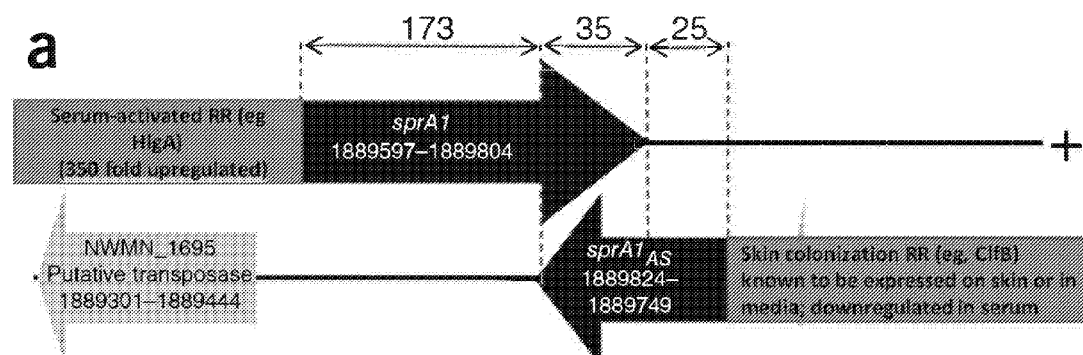


FIG. 1

(57) Abstract: Methods and compositions are provided for durably influencing microbiological ecosystems (microbiomes) in a subject in order to prevent infection and reduce recurrence of infection by microorganisms. In some embodiments, compositions and methods are provided for the creation and use of molecularly-modified bacterial strains with the potential to prevent a variety of microorganism infections.

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METHODS AND COMPOSITIONS TO PREVENT MICROBIAL INFECTION

CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application is being filed on 04 December 2018 as a PCT International Patent application, and claims the benefit of priority to U.S. Provisional Application No. 62/594,943, filed 05 December 2017, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The present application includes a Sequence Listing in electronic format as a txt file entitled "Sequence-Listing," which was created on 03 December 2018 and which has a size of 128,065 kilobytes (KB). The contents of txt file "Sequence-Listing" are incorporated by reference herein.

BACKGROUND OF THE DISCLOSURE

FIELD OF THE DISCLOSURE

[0003] Methods and compositions are provided for durably influencing microbiological ecosystems (microbiomes) in a subject in order to resist infection and reduce recurrence of infection by an undesirable microorganism by decolonizing and durably replacing with a synthetic microorganism. Synthetic microorganisms are provided that may durably replace an undesirable microorganism under dermal or mucosal conditions, and that contain molecular modifications designed to enhance safety, for example, by self-destructing when exposed to systemic conditions, by reducing the potential for acquisition of virulence or antibiotic resistance genes, and/or by producing a desirable product at the site of the ecosystem in a subject.

DESCRIPTION OF THE RELATED ART

[0004] Health care or community associated infection often results from colonizing microorganisms that overcome patient defenses. Inappropriate use of antibiotics may lead to mismanagement of the microbiome. One critical unintended consequence of the mismanagement of the microbiome has been the emergence of antibiotic resistant microorganisms.

[0005] Each individual is host to a vast population of trillions of microorganisms, composed of perhaps 10,000 different species, types and strains. These “commensal” organisms are found both on external sites (e.g. dermal) and on internal sites (e.g. gastrointestinal), and are necessary for survival of the human species. “Colonization” happens automatically through ongoing interactions with the environment.

[0006] The menagerie of microorganisms constitutes the "biome", a dynamic, structured, living system that in many cases, and in many ways, is essential for our health and wellness. A biomic structure is created by a vast combinatorial web of relationships between the host, the environment, and the components of the biome. The human microbiome is an ecosystem. It has a dynamic but persistent structure – it is “resilient” and has a “healthy” normal base state.

[0007] Nonetheless, under some circumstances the microbiome can be invaded and occupied by pathogenic microorganisms. This type of “colonization” may become a precursor to “infection”. This kind of disruption to the microbiome can cause serious and even life-threatening disease.

[0008] One unintended consequence of the mismanagement of our biome has been the emergence of “antibiotic resistance”. This happens when antibiotics and antiseptics do not fully eliminate the target microorganisms. The few survivors that show resistance to these materials then preferentially grow back (“recolonize”) into an open environment (or vacated “niche”) already cleared of competing organisms. The survivor organisms then dominate the space, usually retaining that resistance for their descendants. If exposed to a new killing agent they will tend to develop resistance to that as well. Over only a few generations these microorganisms can develop resistance to many or all of our known antibiotics, becoming the now famous “super-bugs”, and along the way creating an enormous new global health problem.

[0009] A phenomenon called “recurrence” is at the heart of the process that creates antibiotic resistance. While methods to treat pathogenic infection exist, methods to prevent recurrence are effectively nonexistent.

[0010] Bacterial infections are the home territory of the emerging “super bug” phenomenon. The overuse and misuse of antibiotics has caused many strains of pathogenic bacteria to evolve resistance to an increasing number of antibiotic therapies, creating a massive global public health problem. As each new variation of antibiotic is

applied to treat these superbugs, the inevitable process of selecting for resistant strains begins anew, and resistant variants of the pathogen quickly develop. Unfortunately, today bacteria are becoming resistant faster than new antibiotics can be developed.

[0011] Beyond cultivating antibiotic resistance, and frequently causing adverse health effects in the recipients, antibiotic treatments also have the undesirable effect of disrupting the entire microbiome, including both good and bad bacteria. This often creates new problems such as opening the microbiome to colonization by adventitious pathogens after the treatment.

[0012] Bacteria however have less leeway to adapt to different resources, as these requirements are more basic on a molecular level and are intrinsically defined in the genome. This allows the microbiome ecology to behave as more of an “ideal” system, leading to full exclusion of one of the identical strain competitors from the niche.

[0013] The community of organisms colonizing the human body is referred to as the microbiome. The microbiome is often subdivided for analysis into sections of geography (i.e. the skin microbiome versus the gastrointestinal microbiome) or of phylogeny (i.e. bacterial microbiome versus the fungal or protist microbiome).

[0014] Antibiotics are life-saving medicines, but they can also change, unbalance, and disrupt the microbiome. The microbiome is a community of naturally-occurring germs in and on the body—on skin, gut, mouth or respiratory tract, and in the urinary tracts. A healthy microbiome helps protect from infection. Antibiotics disrupt the microbiome, eliminating both “good” and “bad” bacteria. Drug-resistant bacteria—like MRSA, CRE, and *C. difficile*—can take advantage of this disruption and multiply. With this overgrowth of resistant bacteria, the body is primed for infection. Once subjects are colonized with resistant bacteria, the resistant bacteria can easily be spread to others. See “Antibiotic Resistance (AR) Solutions Initiative: Microbiome, CDC Microbiome Fact Sheet 2016”. www.cdc.gov/drugresistance/solutions-initiative/innovations-to-slow-AR.html.

[0015] According to the Center for Disease Control (CDC), the top drug-resistant threats to the United States include *Neisseria gonorrhoeae*, multi-drug resistant *Acinetobacter*, drug-resistant *Campylobacter*, fluconazole-resistant *Candida*, vancomycin-resistant *Enterococcus* (VRE), multi-drug resistant *Pseudomonas Aeruginosa*, drug-resistant non-typhoidal *Salmonella*, drug-resistant *Salmonella*

serotype typhi, methicillin-resistant *Staphylococcus aureus* (MRSA), drug-resistant *Streptococcus pneumoniae*, drug-resistant Tuberculosis, vancomycin-resistant *Staphylococcus aureus*, erythromycin-resistant Group A Streptococcus, and clindamycin-resistant Group B Streptococcus. See “Antibiotic/Antimicrobial Resistance (AR/AMR)”, https://www.cdc.gov/drugresistance/biggest_threats.html.

[0016] *Neisseria gonorrhoeae* causes gonorrhea, a sexually transmitted disease that can result in discharge and inflammation at the urethra, cervix, pharynx, or rectum. There are about 820,000 gonorrhea infections per year. Of these, there are about 246,000 drug-resistant gonorrhea infections: 188,600 tetracycline resistant, 11,489 reduced susceptibility to cefixime, 3,280 reduced susceptibility to ceftriaxone, and about 2,460 exhibit reduced susceptibility to azithromycin.

[0017] *Acinetobacter* is a type of gram-negative bacteria that is a cause of pneumonia or bloodstream infections among critically ill patients. Many of these bacteria have become very resistant to antibiotics. There are about 12,000 *Acinetobacter* infections per year, including about 7,300 multidrug-resistant *Acinetobacter* infections and 500 deaths.

[0018] Candidiasis is a fungal infection caused by yeasts of the genus *Candida*. There are more than 20 species of *Candida* yeasts that can cause infection in humans, the most common of which is *Candida albicans*. *Candida* yeasts normally live on the skin and mucous membranes without causing infection. However, overgrowth or invasion of these microorganisms can cause symptoms to develop. Symptoms of candidiasis vary depending on the area of the body that is infected. *Candida* is the fourth most common cause of healthcare-associated bloodstream infections in the United States. In some hospitals it is the most common cause. These infections tend to occur in the sickest patients. There are about 46,000 *Candida* infections per year, including about 3,400 fluconazole-resistant *Candida* infections, and 220 deaths.

[0019] *Staphylococcus aureus* is a common type of bacteria that is found on the skin. During medical procedures when patients require catheters or ventilators or undergo surgical procedures, *Staphylococcus aureus* can enter the body and cause infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a range of illnesses, from skin and wound infections to pneumonia and bloodstream infections that can cause sepsis and death. Staph bacteria, including MRSA, are one of the most common causes of healthcare-associated infections. There are about 80,461 severe MRSA infections per

year, and about 11,285 deaths from MRSA per year. When *Staphylococcus aureus* becomes resistant to vancomycin, there are few treatment options available because vancomycin-resistant *Staphylococcus aureus* bacteria identified to date were also resistant to methicillin and other classes of antibiotics. There have been at least 13 cases of vancomycin-resistant *Staphylococcus aureus* in 4 states since 2002.

[0020] *Streptococcus pneumoniae* (*S. pneumoniae*, or pneumococcus) is the leading cause of bacterial pneumonia and meningitis in the United States. It also is a major cause of bloodstream infections and ear and sinus infections. There are about 1,200,000 drug resistant infections per year, with about 19,000 excess hospitalizations, and 7,000 deaths.

[0021] Group A Streptococcus (GAS) causes many illnesses, including pharyngitis (strep throat), streptococcal toxic shock syndrome, necrotizing fasciitis (“flesh-eating” disease), scarlet fever, rheumatic fever, and skin infections such as impetigo. Group A Streptococcus is the leading cause of necrotizing fasciitis (“flesh-eating” disease). There are about 1-2.6 million Strep throat infections per year, including about 1,300 drug-resistant Group A Strep infections per year, and about 160 deaths.

[0022] Group B Streptococcus (GBS) is a type of bacteria that can cause severe illness in people of all ages, ranging from bloodstream infections (sepsis) and pneumonia to meningitis and skin infections. Group B Strep is the leading cause of serious microorganism infections in newborns. There were about 27,000 severe cases of GBS in 2011, including about 7,600 drug-resistant Group B Strep infections, and about 440 deaths.

[0023] Prior art methods of preventing infection and transmission of drug-resistant microorganisms in colonized individuals include screening and isolation, decolonization of the drug-resistant microorganism, and/or recolonization with a drug-susceptible microorganism.

[0024] Prior art methods employing suppression (decolonization) alone –such as use of antibiotics and antimicrobial agents- often fail because they are subject to high rates of recurrence. Decolonization is often insufficient when used alone to effectively prevent recurrence and/or transmission of the drug-resistant microorganism.

[0025] Among pathogenic microorganisms causing health care related infection, methicillin-resistant *Staphylococcus aureus* (MRSA) has been given priority because of its virulence and disease spectrum, multidrug resistant profile and increasing prevalence

in health care settings. MRSA is the most common cause of ventilator-associated pneumonia and surgical site infection and the second most common cause of central catheter associated bloodstream infection.

[0026] Strategies involving screening of new hospital patients for MRSA, and isolating those who carry it, with or without decolonization have been shown to be somewhat effective in reducing transmission. However, this type of therapy is rather expensive requiring extra accommodations, with special containment and hygiene procedures.

[0027] Decolonization alone has been used in hospital patients in an attempt to reduce transmission and prevent disease in *Staphylococcus aureus* carriers. Decolonization may involve a multi-day regimen of antibiotic and/or antiseptic agents- for example, intranasal mupirocin and chlorhexidine bathing. Universal decolonization is a method employed by some hospitals where all intensive care unit (ICU) hospital patients are washed daily with chlorhexidine and intranasal mupirocin, but since its widespread use, MRSA infection rates in the U.S. have not significantly changed. In addition, microorganisms may develop resistance to chlorhexidine and mupirocin upon repeated exposure.

[0028] Decolonization when used alone may not be durable because the vacated niche may become recolonized with pathogenic or drug-resistant microorganisms.

[0029] For example, Shinefield et al., 1963, Amer J Dis Child 105, June 1963, 146-154, observed that colonization of newborn infants with strains of *Staphylococcus aureus* of the 52/52a/80/81 phage complex by contact with a carrier was often followed by disease in babies and their family contacts. Shinefield also observed that control measures using antiseptic or antimicrobial agents applied to the infant lead to colonization with abnormal flora, consisting primarily of highly resistant coagulase negative staphylococci and Gram-negative organisms such as *Pseudomonas* and *Proteus*. Shinefield attempted to solve the problem by artificially colonizing newborns with staphylococcal strain 502a by nasal and/or umbilical inoculation. 502a is a coagulase positive strain of *Staphylococcus aureus* of low virulence, susceptible to penicillin, and incapable of being induced to produce beta-lactamase. It was shown that presence of other staphylococci interfered with acquisition of 502a. Persistence of colonization was at best 35% after 6 months to one year.

[0030] Boris M. et al., "Bacterial Interference: Protection Against Recurrent Intrafamilial Staphylococcal Disease." *Amer J Dis Child* 115 (1968): 521-29, deliberately colonized ~4000 infants in first few hours of life with *Staphylococcus aureus* 502a (nares & umbilical stump). Virtually complete protection of babies from 80/81 infection was observed (babies were monitored for 1-year post inoculation). Although 5-15% of babies developed tiny treatment emergent vesicles that self-resolved in first 3 days post-treatment. Prior decolonization improves persistence of 502a up to 5-fold compared to placebo (saline) n=63. Controlled studies in recurrent furunculosis showed that decolonization with systemic antibiotics + nasal antimicrobial followed by application of 502a curtailed disease in 80% of patients.

[0031] Recolonization with a drug-susceptible strain may not be safe because the drug-susceptible strain may still cause systemic infection.

[0032] Shinefield et al., 1973, *Microbiol Immunol*, vol. 1, 541-547, reported using bacterial replacement including decolonization in treating patients with recurrent furunculosis. Chronic staphylococcal carriers were treated with antibiotic therapy including systemic antibiotics and application of antimicrobial cream to nasal mucosa. In an initial study, 31 patients received antibiotic therapy alone and exhibited a 74% recurrence rate of original strain. 18 patients received antibiotic treatment followed by 502a inoculation and exhibited 27% recurrence of original strain. A larger study of 587 patients resulted in 21% recurrence of original strain after 12 months. However, a high relapse rate was noted in patients with diabetes, eczema or acne. Disease associated with 502a was noted in 11 patients.

[0033] Aly et al., 1974 *J Infect Dis* 129(6) pp. 720-724, studied bacterial interference in carriers of *Staphylococcus aureus*. The carriers were treated with antibiotics and antibacterial soaps and challenged with strain 502a. Specifically, decolonization method involved oral dicloxacillin 8 days; neosporin in nose for 8 days, and trichlorocabanilide. It was found that full decolonization was needed to get good take. Day 7 showed 100% take, but at day 23 the take was down to 60 to 80%. The persistence data was 73% at 23 weeks for well-decolonized subjects, and only 17% persistence for partially decolonized subjects. Co-colonization was found in 5/12 subjects at day 3, 2/12 subjects at day 10, and 1/12 subjects at day 35 and at day 70.

[0034] Decolonization, followed by recolonization with a microorganism of the same genus, but a different species, may not be durable because the vacated niche is not adequately filled by the different species.

[0035] WO2009117310 A2, George Liu, assigned to Cedars-Sinai Medical Center, discloses methods for treatment and prevention of methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *Staphylococcus aureus* (MSSA) using a decolonization/recolonization method. In one example, mice are treated with antibiotics to eradicate existing flora, including MRSA, and newly cleared surface area is colonized with bacteria of the same genus, but of a different species, such as *Staphylococcus epidermidis*. No specific data regarding recurrence is provided.

[0036] Administration of probiotics in an attempt to treat infection by pathogenic microorganisms may not be effective and may not be durable because the probiotic may not permanently colonize the subject.

[0037] U.S. Pat. No. 6,660,262, Randy McKinney, assigned to Bovine Health Products, Inc., discloses broad spectrum antimicrobial compositions comprising certain minerals, vitamins, cobalt amino acids, kelp and a *Lactobacillus* species for use in treating microbial infection in animals. Field trials in cattle and horses were performed, but the infectious bacterial strain or other infectious agent was not identified.

[0038] U.S. Pat. No. 6,905,692, Sean Farmer, assigned to Ganeden Biotech, Inc., discloses topical compositions containing certain combinations of probiotic *Bacillus* bacteria, spores and extracellular products for application to skin or mucosa of a mammal for inhibiting growth of certain bacterium, yeast, fungi, and virus. Compositions comprising *Bacillus coagulans* spores, or *Bacillus* species. culture supernatants and *Pseudomonas lindbergii* culture supernatants in a vehicle such as emu oil are provided. The disclosure states since probiotics do not permanently colonize the host, they need to be ingested or applied regularly for any health-promoting properties to persist.

[0039] U.S. Pat. No. 6,461,607, Sean Farmer, assigned to Ganeden Biotech, Inc., discloses lactic acid-producing bacteria, preferably strains of *Bacillus coagulans*, for the control of gastrointestinal tract pathogens in a mammal. Methods for selective breeding and isolation of probiotic, lactic acid-producing bacterial strains which possess resistance to an antibiotic are disclosed. Methods for treating infections with a composition

comprising an antibiotic-resistant lactic-acid producing bacteria and an antibiotic are disclosed.

[0040] U.S. Pat. No. 8,906,668, assigned to Seres Therapeutics, provide cytotoxic binary combinations of 2 or more bacteria of different operational taxonomic units (OTUs) to durably exclude a pathogenic bacterium. The OTUs are determined by comparing sequences between organisms, for example as sharing at least 95% sequence identity of 16S ribosomal RNA genes in at least in a hypervariable region.

[0041] Prior art methods employing replacement of the original pathogenic microorganism (recolonization) alone are subject to poor colonization rates with the new microorganism. The process may fail if the recolonization is done incorrectly. Effective recolonization is critical but not sufficient when used alone to prevent recurrence.

[0042] Prior art methods involving both suppression (decolonization) of the original pathogenic microorganism and replacement (recolonization) with a new microorganism may give variable recurrence of the pathogenic microorganism depending on the specific method.

[0043] Rather than waging an un-winnable war against commensal pathogenic or drug-resistant microorganisms, a better approach may be to manage the microbiome: to actively promote "good bugs" and their supporting system dynamics, while selectively suppressing the recurrence of specific pathogenic organisms. Improved methods to safely and durably prevent and reduce recurrence of infection by undesirable microorganisms, such as virulent, pathogenic and/or drug-resistant microorganisms, are desirable.

SUMMARY OF THE INVENTION

[0044] Methods and compositions are provided for safely and durably influencing microbiological ecosystems (microbiomes) in a subject to perform a variety of functions, for example, including reducing the risk of infection by an undesirable microorganism such as virulent, pathogenic and/or drug-resistant microorganism.

[0045] Methods are provided herein to prevent or reduce the risk of colonization, infection, recurrence of colonization, or recurrence of a pathogenic infection by an undesirable microorganism in a subject, comprising: decolonizing the undesirable microorganism on at least one site in the subject to reduce or eliminate the presence of the undesirable microorganism from the site; and durably replacing the undesirable

microorganism by administering a synthetic microorganism to the at least one site in the subject, wherein the synthetic microorganism can durably integrate with a host microbiome by occupying the niche previously occupied by the undesirable microorganism; and optionally promoting colonization of the synthetic microorganism within the subject.

[0046] The disclosure provides a method for eliminating and preventing the recurrence of a undesirable microorganism in a subject hosting a microbiome, comprising (a) decolonizing the host microbiome; and (b) durably replacing the undesirable microorganism by administering to the subject a synthetic microorganism comprising at least one element imparting a non-native attribute, wherein the synthetic microorganism is capable of durably integrating to the host microbiome, and occupying the same niche in the host microbiome as the undesirable microorganism.

[0047] In some embodiments, the decolonizing is performed on at least one site in the subject to substantially reduce or eliminate the detectable presence of the undesirable microorganism from the at least one site.

[0048] In some embodiments, the detectable presence of an undesirable microorganism or a synthetic microorganism is determined by a method comprising a phenotypic method and/or a genotypic method, optionally wherein the phenotypic method is selected from the group consisting of biochemical reactions, serological reactions, susceptibility to anti-microbial agents, susceptibility to phages, susceptibility to bacteriocins, and/or profile of cell proteins. In some embodiments, the genotypic method is selected a hybridization technique, plasmids profile, analysis of plasmid polymorphism, restriction enzymes digest, reaction and separation by Pulsed-Field Gel Electrophoresis (PFGE), ribotyping, polymerase chain reaction (PCR) and its variants, Ligase Chain Reaction (LCR), and Transcription-based Amplification System (TAS).

[0049] In some embodiments, the niche is a dermal or mucosal environment that allows stable colonization of the undesirable microorganism at the at least one site in the subject.

[0050] In some embodiments, the ability to durably integrate to the host microbiome is determined by detectable presence of the synthetic microorganism at the at least one site for a period of at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least

30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

[0051] In some embodiments, the ability to durably replace the undesirable microorganism is determined by the absence of detectable presence of the undesirable microorganism at the at least one site for a period of at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

[0052] In some embodiments, the ability to occupy the same niche is determined by absence of co-colonization of the undesirable microorganism and the synthetic microorganism at the at least one site after the administering step. In some embodiments, the absence of co-colonization is determined at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

[0053] In some embodiments, the synthetic microorganism comprises at least one element imparting the non-native attribute that is durably incorporated to the synthetic microorganism. In some embodiments, the at least one element imparting the non-native attribute is durably incorporated to the host microbiome via the synthetic microorganism.

[0054] In some embodiments, the at least one element imparting the non-native attribute is a kill switch molecular modification, virulence block molecular modification, or nanofactory molecular modification. In some embodiments, the synthetic microorganism comprises molecular modification that is integrated to a chromosome of the synthetic microorganism. In some embodiments, the synthetic microorganism comprises a virulence block molecular modification that prevents horizontal gene transfer of genetic material from the undesirable microorganism.

[0055] In some embodiments, the measurable average cell death of the synthetic microorganism occurs within at least a preset period of time following induction of the first promoter after the change in state. In some embodiments, the measurable average cell death occurs within at least a preset period of time selected from the group consisting of within at least 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, or 360 min minutes following the change of state. In some embodiments, the measurable average cell death is at least a

50% cfu, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% cfu count reduction following the preset period of time. In some embodiments, the change in state is selected from one or more of pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, metal concentration, chelated metal concentration, change in composition or concentration of one or more immune factors, mineral concentration, and electrolyte concentration. In some embodiments, the change in state is a higher concentration of and/or change in composition of blood, serum, or plasma compared to normal physiological (niche) conditions at the at least one site in the subject.

[0056] In some embodiments, the undesirable microorganism is a *Staphylococcus aureus* strain, and wherein the detectable presence is measured by a method comprising obtaining a sample from the at least one site of the subject, contacting a chromogenic agar with the sample, incubating the contacted agar and counting the positive cfus of the bacterial species after a predetermined period of time.

[0057] In some embodiments, a method is provided comprising a decolonizing step comprising topically administering a decolonizing agent to at least one site in the subject to reduce or eliminate the presence of the undesirable microorganism from the at least one site.

[0058] In some embodiments, the decolonizing step comprises topical administration of a decolonizing agent, wherein no systemic antimicrobial agent is simultaneously administered. In some embodiments, no systemic antimicrobial agent is administered prior to, concurrent with, and/or subsequent to within one week, two weeks, three weeks, one month, two months, three months, six months, or one year of the first topical administration of the decolonizing agent or administration of the synthetic microorganism. In some embodiments, the decolonizing agent is selected from the group consisting of a disinfectant, bactericide, antiseptic, astringent, and antimicrobial agent. In some embodiments, the decolonizing agent is selected from the group consisting of alcohols (ethyl alcohol, isopropyl alcohol), aldehydes (glutaraldehyde, formaldehyde, formaldehyde-releasing agents (noxythiolin = oxymethylenethiourea, tauroline, hexamine, dantoin), o-phthalaldehyde), anilides (triclocarban = TCC = 3,4,4'-trichlorocarbanilide), biguanides (chlorhexidine, alexidine, polymeric biguanides (polyhexamethylene biguanides with MW> 3,000 g/mol, vantocil), diamidines

(propamidine, propamidine isethionate, propamidine dihydrochloride, dibromopropamidine, dibromopropamidine isethionate), phenols (fentichlor, p-chloro-m-xyleneol, chloroxylenol, hexachlorophene), bis-phenols (triclosan, hexachlorophene), chloroxylenol (PCMX), quaternary ammonium compounds (cetrimide, benzalkonium chloride, cetyl pyridinium chloride), silver compounds (silver sulfadiazine, silver nitrate), peroxy compounds (hydrogen peroxide, peracetic acid, benzoyl peroxide), iodine compounds (povidone-iodine, poloxamer-iodine, iodine), chlorine-releasing agents (sodium hypochlorite, hypochlorous acid, chlorine dioxide, sodium dichloroisocyanurate, chloramine-T), copper compounds (copper oxide), isotretinoin, sulfur compounds, botanical extracts (*Melaleuca* spp. (tea tree oil), (*Vaccinium* spp. (e.g., A-type proanthocyanidins), *Cassia fistula* Linn, *Baekea frutesdens* L., *Melia azedarach* L., *Muntingia calabura*, *Vitis vinifera* L., *Terminalia avicennioides* Guill & Perr., *Phyllanthus discoideus* Muel. Muel-Arg., *Ocimum gratissimum* Linn., *Acalypha wilkesiana* Muell-Arg., *Hypericum pruinatum* Boiss.&Bal., *Hypericum olympicum* L. and *Hypericum sabrum* L., *Hamamelis virginiana* (witch hazel), Clove oil, *Eucalyptus* spp., *rosmarinus officinalis* spp.(rosemary), *thymus* spp.(thyme), *Lippia* spp. (oregano), lemongrass spp., *cinnamomum* spp., *geranium* spp., *lavendula* spp.), aminolevulonic acid, and topical antibiotic compounds (bacteriocins; mupirocin, bacitracin, neomycin, polymyxin B, gentamicin).

[0059] In some embodiments, the antimicrobial agent is selected from the group consisting of vancomycin, cefazolin, cephalothin, cephalixin, linezolid, daptomycin, clindamycin, lincomycin, mupirocin, bacitracin, neomycin, polymyxin B, gentamicin, prulifloxacin, ulifloxacin, fidaxomicin, minocyclin, metronidazole, metronidazole, sulfamethoxazole, ampicillin, trimethoprim, ofloxacin, norfloxacin, tinidazole, norfloxacin, ornidazole, levofloxacin, nalidixic acid, ceftriaxone, azithromycin, cefixime, ceftriaxone, cefalexin, ceftriaxone, rifaximin, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, gatifloxacin, gemifloxacin, prufloxacin, ulifloxacin, moxifloxacin, nystatin, amphotericin B, flucytosine, ketoconazole, posaconazole, clotrimazole, voriconazole, griseofulvin, miconazole nitrate, and fluconazole.

[0060] In some embodiments, the decolonizing comprises topically administering the decolonizing agent at least one, two, three, four, five or six or more times prior to the replacing step. In some embodiments, the decolonizing step comprises administering the decolonizing agent to the at least one host site in the subject from one to six or more

times or two to four times at intervals of between 0.5 to 48 hours apart, and wherein the replacing step is performed after the final decolonizing step.

[0061] In some embodiments, a method is provided comprising decolonizing an undesirable microorganism, and replacing with a synthetic microorganism comprising topical administration of a composition comprising at least 10^5 , at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} , or at least 10^{11} CFU of the synthetic strain and a pharmaceutically acceptable carrier to at least one host site in the subject. In some embodiments, the initial replacing step is performed within 12 hours, 24 hours, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days, or between 0.5-10 days, 1-7 days, or 2 to 5 days of the decolonizing step. In some embodiments, the replacing step is repeated at intervals of no more than once every two weeks to six months following the final decolonizing step. In some embodiments, the decolonizing step and the replacing step is repeated at intervals of no more than once every two weeks to six months, or three weeks to three months. In some embodiments, the replacing comprises administering the synthetic microorganism to the at least one site at least one, two, three, four, five, six, seven, eight, nine, or ten times. In some embodiments, the replacing comprises administering the synthetic microorganism to the at least one site no more than one, no more than two, no more than three times, or no more than four times per month.

[0062] In some embodiments, the method of decolonizing the undesirable microorganism and replacing with a synthetic microorganism further comprises promoting colonization of the synthetic microorganism in the subject. In some embodiments, the promoting colonization of the synthetic microorganism in the subject comprises administering to the subject a promoting agent, optionally where the promoting agent is a nutrient, prebiotic, commensal, stabilizing agent, humectant, and/or probiotic bacterial species. In some embodiments, the promoting comprises administering a probiotic species at from 10^5 to 10^{10} cfu, 10^6 to 10^9 cfu, or 10^7 to 10^8 cfu to the subject after the initial decolonizing step.

[0063] In some embodiments, the nutrient is selected from sodium chloride, lithium chloride, sodium glycerophosphate, phenylethanol, mannitol, tryptone, peptide, and yeast extract. In some embodiments, the prebiotic is selected from the group consisting of short-chain fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid), glycerol, pectin-derived oligosaccharides from agricultural by-

products, fructo-oligosaccharides (e.g., inulin-like prebiotics), galacto-oligosaccharides (e.g., raffinose), succinic acid, lactic acid, and mannan-oligosaccharides.

[0064] In some embodiments, the probiotic is selected from the group consisting of *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Streptococcus thermophiles*, and *Enterococcus faecalis*.

[0065] In some embodiments, the commensal is selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*.

[0066] In some embodiments, the undesirable microorganism is an antimicrobial agent-resistant microorganism. In some embodiments, the antimicrobial agent-resistant microorganism is an antibiotic resistant bacteria. In some embodiments, the antibiotic-resistant bacteria is a Gram-positive bacterial species selected from the group consisting of a *Streptococcus* spp., *Cutibacterium* spp., and a *Staphylococcus* spp. In some embodiments, the *Streptococcus* spp. is selected from the group consisting of *Streptococcus pneumoniae*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*. In some embodiments, the *Cutibacterium* spp. is selected from the group consisting of *Cutibacterium acnes* subsp. *acnes*, *Cutibacterium acnes* subsp. *defendens*, and *Cutibacterium acnes* subsp. *elongatum*. In some embodiments, the *Staphylococcus* spp. is selected from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus*. In some embodiments, the undesirable microorganism is a methicillin-resistant

Staphylococcus aureus (MRSA) strain that contains a staphylococcal chromosome cassette (SCC*mec* types I-III), which encode one (SCC*mec* type I) or multiple antibiotic resistance genes (SCC*mec* type II and III), and/or produces a toxin. In some embodiments, the toxin is selected from the group consisting of a Panton-Valentine leucocidin (PVL) toxin, toxic shock syndrome toxin-1 (TSST-1), staphylococcal alpha-hemolysin toxin, staphylococcal beta-hemolysin toxin, staphylococcal gamma-hemolysin toxin, staphylococcal delta-hemolysin toxin, enterotoxin A, enterotoxin B, enterotoxin C, enterotoxin D, enterotoxin E, and a coagulase toxin.

[0067] In some embodiments, the subject treated with a method according to the disclosure does not exhibit recurrence or colonization of the undesirable microorganism as evidenced by swabbing the subject at the at least one site for at least two weeks, at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 24 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

[0068] The disclosure provides a synthetic microorganism for durably replacing an undesirable microorganism in a subject. The synthetic microorganism comprises a molecular modification designed to enhance safety by reducing the risk of systemic infection. In one embodiment, the molecular modification causes a significant reduction in growth or cell death of the synthetic microorganism in response to blood, serum, or plasma. The synthetic microorganism may be used in methods and compositions for preventing or reducing recurrence of dermal or mucosal colonization or recolonization of an undesirable microorganism in a subject.

[0069] The disclosure provides a synthetic microorganism for use in compositions and methods for treating or preventing, reducing the risk of, or reducing the likelihood of colonization, or recolonization, systemic infection, bacteremia, or endocarditis caused by an undesirable microorganism in a subject.

[0070] The disclosure provides a synthetic microorganism comprising a recombinant nucleotide comprising at least one kill switch molecular modification comprising a first cell death gene operatively associated with a first regulatory region comprising an inducible first promoter, wherein the first inducible promoter exhibits conditionally high level gene expression of the recombinant nucleotide in response to exposure to blood, serum, or plasma of at least three fold increase of basal productivity. In some

embodiments, the inducible first promoter exhibits, comprises, is derived from, or is selected from a gene that exhibits upregulation of at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold within at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, or at least 360 min following exposure to blood, serum, or plasma.

[0071] In some embodiments, the synthetic microorganism comprises a kill switch molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising an inducible first promoter, wherein the first promoter is activated (induced) by a change in state in the microorganism environment in contradistinction to the normal physiological (niche) conditions at the at least one site in the subject.

[0072] In some embodiments, the synthetic microorganism further comprises an expression clamp molecular modification comprising an antitoxin gene specific for the first cell death gene or a product thereof, wherein the antitoxin gene is operably associated with a second regulatory region comprising a second promoter which is constitutive or active upon dermal or mucosal colonization or in a complete media, but is not induced, induced less than 1.5-fold, or is repressed after exposure to blood, serum or plasma for at least 30 minutes. In some embodiments, the second promoter is active upon dermal or mucosal colonization or in TSB media, but is repressed by at least 2 fold upon exposure to blood, serum or plasma after a period of time of at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, or at least 360 min.

[0073] In some embodiments, the synthetic microorganism exhibits measurable average cell death of at least 50% cfu reduction within at least 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, or 360 minutes following exposure to blood, serum, or plasma. In some embodiments, the synthetic microorganism exhibits measurable average cell death of at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% cfu count reduction within at least 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, or 360 minutes following exposure to blood, serum, or plasma.

[0074] In some embodiments, the synthetic microorganism comprises a kill switch molecular modification that reduces or prevents infectious growth of the synthetic microorganism under systemic conditions in a subject.

[0075] In some embodiments, the synthetic microorganism comprises at least one molecular modification that is integrated to a chromosome of the synthetic microorganism.

[0076] In some embodiments, the synthetic microorganism is derived from a target microorganism having the same genus and species as an undesirable microorganism. In some embodiments, the target microorganism is susceptible to at least one antimicrobial agent. In some embodiments, the target microorganism is selected from a bacterial, fungal, or protozoal target microorganism. In certain embodiments, the target microorganism is capable of colonizing a dermal and/or mucosal niche.

[0077] In some embodiments, the target microorganism has the ability to biologically integrate with the decolonized host microbiome. In some embodiments, the synthetic microorganism is derived from a target microorganism isolated from the host microbiome. In some embodiments, the target microorganism is selected from a bacterial, fungal, or protozoal target microorganism.

[0078] In some embodiments, the target microorganism that is a member of a genus selected from the group consisting of *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Pseudomonas*.

[0079] In some embodiments, the target microorganism is a bacterial species capable of colonizing a dermal and/or mucosal niche and is a member of a genus selected from the group consisting of *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Pseudomonas*. In some embodiments, the target microorganism is selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*. In a particular embodiment, a synthetic microorganism is provided that is

derived from a *Staphylococcus aureus* strain. In some embodiments, the target strain is a *Staphylococcus aureus* 502a strain or RN4220 strain.

[0080] In some embodiments, the synthetic microorganism comprises a kill switch molecular modification comprising a cell death gene selected from the group consisting of *sprA1*, *sprA2*, *kpn1*, *sma1*, *sprG*, *relF*, *rsaE*, *yoeB*, *mazF*, *yefM*, or lysostaphin toxin gene. In some embodiments, the cell death gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 122, 124, 125, 126, 127, 128, 274, 275, 284, 286, 288, 290, 315, and 317, or a substantially identical nucleotide sequence.

[0081] In some embodiments, the inducible first promoter is a blood, serum, and/or plasma responsive promoter. In some embodiments, the first promoter is upregulated by at least 1.5 fold, at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold within a period of time selected from the group consisting of at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, and at least 360 min following exposure to human blood, serum or plasma. In some embodiments, the first promoter is not induced, induced less than 1.5 fold, or is repressed in the absence of the change of state. In some embodiments, the first promoter is induced at least 1.5, 2, 3, 4, 5 or at least 6 fold within a period of time in the presence of serum, blood or plasma. In some embodiments, the first promoter is not induced, induced less than 1.5 fold, or repressed under the normal physiological (niche) conditions at the at least one site.

[0082] In some embodiments, the inducible first promoter comprises or is derived from a gene selected from the group consisting of *isdA* (iron-regulated surface determinant protein A), *isdB* (iron-regulated surface determinant protein B), *isdG* (heme-degrading monooxygenase), *hlgA* (gamma-hemolysin component A), *hlgA1* (gamma-hemolysin), *hlgA2* (gamma-hemolysin), *hlgB* (gamma-hemolysin component B), *hrtAB* (heme-regulated transporter), *sbnC* (luc C family siderophore biosynthesis protein), *sbnD*, *sbnI*, *sbnE* (lucA/lucC family siderophore biosynthesis protein), *isdI*, *lrgA* (murein hydrolase regulator A), *lrgB* (murein hydrolase regulator B), *ear* (Ear protein), *fhuA* (ferrichrome transport ATP-binding protein fhuA), *fhuB* (ferrichrome transport permease), *hly* (phospholipase C), heme ABC transporter 2 gene, heme ABC transporter gene, *isd* ORF3, *sbnF*, alanine dehydrogenase gene, diaminopimelate decarboxylase gene, iron ABC transporter gene, threonine dehydratase gene, siderophore ABC transporter gene, SAM dep Metrans gene, *HarA*, *splF* (serine protease SplF), *splD* (serine protease SplD), *dps* (general stress protein 20U), SAUSA300_2617 (putative cobalt ABC transporter,

ATP-binding protein), SAUSA300_2268 (sodium/bile acid symporter family protein), SAUSA300_2616 (cobalt family transport protein), *srtB* (Sortase B), *sbnA* (probable siderophore biosynthesis protein sbnA), *sbnB*, *sbnG*, *leuA* (2-isopropylmalate synthase amino acid biosynthetic enzyme), *sstA* (iron transport membrane protein), *sirA* (iron ABC transporter substrate-binding protein), *isdA* (heme transporter), and *spa* (Staphylococcal protein A). In some embodiments, the inducible first promoter comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, and 163, or a substantially identical nucleotide sequence thereof.

[0083] In some embodiments, the synthetic microorganism comprises an expression clamp molecular modification comprising a second promoter operatively associated with an antitoxin gene that encodes an antisense RNA sequence capable of hybridizing with at least a portion of the first cell death gene. In some embodiments, the antitoxin gene encodes an antisense RNA sequence capable of hybridizing with at least a portion of the first cell death gene. In some embodiments, the antitoxin gene is selected from the group consisting of a *sprA1* antitoxin gene, *sprA2* antitoxin gene, *sprG* antitoxin gene or *sprF*, *holin* antitoxin gene, *187-lysK* antitoxin gene, *yefM* antitoxin gene, *lysostaphin* antitoxin gene, or *mazE* antitoxin gene, *kpn1* antitoxin gene, *sma1* antitoxin gene, *relF* antitoxin gene, *rsaE* antitoxin gene, or *yoeB* antitoxin gene, respectively. In some embodiments, the antitoxin gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 273, 306, 307, 308, 309, 310, 311, 312, 314, 319, or 322, or a substantially identical nucleotide sequence.

[0084] In some embodiments, the second promoter comprises or is derived from a gene selected from the group consisting of *clfB* (Clumping factor B), *sceD* (autolysin, exoprotein D), *walkR* (virulence regulator), *atlA* (Major autolysin), *oatA* (O-acetyltransferase A); phosphoribosylglycinamide formyltransferase gene, phosphoribosylaminoimidazole synthetase gene, amidophosphoribosyltransferase gene, phosphoribosylformylglycinamide synthase gene, phosphoribosylformylglycinamide synthase gene, phosphoribosylaminoimidazole-succinocarboxamide gene, trehalose permease IIC gen, DeoR family transcriptional regulator gene, phosphofructokinase gene, PTS fructose transporter subunit IIC gene, galactose-6-phosphate isomerase gene, NarZ, NarH, NarT, alkylhydroperoxidase gene,

hypothetical protein gene, DeoR trans factor gene, lysophospholipase gene, protein disaggregation chaperon gene, alkylhydroperoxidase gene, phosphofructokinase gene, *gyrB*, *sigB*, and *rho*. In some embodiments, the second promoter is a P_{clfB} (clumping factor B) that comprises a nucleotide sequence of SEQ ID NO: 117, 118, 129 or 130, or a substantially identical nucleotide sequence thereof.

[0085] In some embodiments, the synthetic microorganism comprises a virulence block molecular modification, and/or a nanofactory molecular modification. In some embodiments, the virulence block molecular modification prevents horizontal gene transfer of genetic material from the undesirable microorganism.

[0086] In some embodiments, the nanofactory molecular modification comprises an insertion of a gene that encodes, a knock out of a gene that encodes, or a genetic modification of a gene that encodes a product selected from the group consisting of an enzyme, amino acid, metabolic intermediate, and a small molecule.

[0087] The disclosure provides a composition comprising an effective amount of a synthetic microorganism according to the disclosure and a pharmaceutically acceptable carrier, diluent, emollient, binder, excipient, lubricant, sweetening agent, flavoring agent, wetting agent, preservative, buffer, or absorbent, or a combination thereof. In some embodiments, the composition further comprises a promoting agent. In some embodiments, the promoting agent is selected from a nutrient, prebiotic, commensal, and/or probiotic bacterial species.

[0088] The disclosure provides a single dose unit comprising a composition or synthetic microorganism of the disclosure. In some embodiments, the single dose unit comprises at least at least about 10^5 , at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} CFU, or at least 10^{11} of the synthetic strain and a pharmaceutically acceptable carrier. In some embodiments, the single dose unit is formulated for topical administration. In some embodiments, the single dose unit is formulated for dermal or mucosal administration.

[0089] The disclosure provides a synthetic microorganism, composition according to the disclosure for use in the manufacture of a medicament for use in a method eliminating, preventing, or reducing the risk of the recurrence of a undesirable microorganism in a subject.

[0090] The disclosure provides a kit for preventing or reducing recurrence of dermal or mucosal colonization or recolonization of an undesirable microorganism in a subject, the

kit comprising in at least one container, comprising a synthetic microorganism, composition, or single dose of the disclosure, and optionally one or more additional components selected from a second container comprising a decolonizing agent, a sheet of instructions, at least a third container comprising a promoting agent, and/or an applicator.

BRIEF DESCRIPTION OF THE DRAWINGS

[0091] FIG. 1 shows a diagram of a representative molecular modification inserted to a *Staphylococcus aureus*, e.g., BioPlx-01, to create a synthetic microorganism BioPlx strain. A cassette comprising the molecular modification comprises a kill switch and an expression clamp, including expression clamp (e.g., ClfB) promoter cloned to drive expression of the SprA1 antisense (antitoxin) RNA wherein the cassette is incorporated into the same expression module from a kill switch comprising a serum-responsive promoter (e.g., *PhlgA*) operably associated with SprA1 toxin gene. In this strain, serum/blood exposure activates the toxin (e.g., up to 350-fold or more) but not the antitoxin, and growth in TSB or on the skin activates antitoxin but not toxin.

[0092] FIG. 2 shows shuttle vector PCN51 used to clone genes into an *E coli* - *Staphylococcus aureus* pass-through strain (IMO8B) for transfection of the vector into BioPlx-01 for evaluation.

[0093] FIGs. 3A-3C shows Table 4A with primer sequences for recombinant construction of synthetic *Staphylococcus aureus* from strain BioPlx-01.

[0094] FIGs. 4A-4D shows Table 4B with primer sequences for CRISPR construction of synthetic *Staphylococcus aureus* from strain BioPlx-01.

[0095] FIG. 5A shows a genetic map of a pKOR1 Integrative Plasmid depicting the *repF* (replication gene of pE194ts), *secY570* (N-terminal 570 nucleotides of secY including ribosome binding site), *cat* (chloramphenicol acetyltransferase), attP (page lambda attachment site), *ori*(-) (ColE1 plasmid replication origin), and *bla* (b-lactamase). (+) or (-) indicates functions in gram positive (+) or gram negative (-) bacteria. The P_{xyl/tetO} promoter and the transcription direction of the promoter are indicated by an arrow.

[0096] FIG. 5B shows a genetic map of a pIMAY Integrative Plasmid. (accession number JQ62198).

[0097] FIG. 6 shows fold-induction of the *HlgA* (gamma hemolysin) promoter candidate in a methicillin-susceptible *Staphylococcus aureus* strain BioPlx-01 by incubation with human serum. Expression was normalized to a housekeeping gene (*gyrB*) and was compared with that in cells growing logarithmically in liquid TSB media.

[0098] FIG. 7 shows fold-induction of the *SstA* (iron transport) promoter candidate in a methicillin-susceptible *Staphylococcus aureus* strain BioPlx-01 by incubation with human serum. Expression was normalized to a housekeeping gene (*GyrB*) and was compared with that in cells growing logarithmically in liquid TSB media.

[0099] FIG. 8 shows CRISPR gRNA target site intergenic region identified between 1,102,100 and 1,102,700 bp in the *Staphylococcus aureus* 502a genome, GenBank: CP007454.1.

[00100] FIG. 9 shows a representative screen shot of CRISPRScan used to find putative gRNAs for use in CRISPR methods.

[00101] FIG. 10 shows cassette for integration via CRISPR and layout of the pCasSA vector. Cap1A is a constitutive promoter controlling gRNA transcription. Target seq is targeting sequence, for example, with 10 possible cutting targets (1.1, 1.2 etc.). sgRNA is single-strand guide RNA (provides structural component). Xba1 and Xho1 are two restriction sites used to add the HA's to the pCasSA vector. HAs are homologous arms to use as templates for homology directed repair (typically 200 – 1000 bp). *P_{rpsL}*-mCherry is a constitutive promoter controlling the “optimized” mCherry. *P_{rpsL}*-Cas9 is a constitutive promoter controlling Cas9 protein expression.

[00102] FIG. 11 shows vectors for use in the present disclosure. A is a vector used for promoter screen with fluorescence using pCN51. B is a vector for promoter screen with cell death gene. C is a vector for chromosomal integration using CRISPR. D is a vector for chromosomal integration using homologous recombination. Left & Right (or upstream and downstream) HA: homology arms to genomic target locus, CRISPR targeting: RNA guide to genomic locus, mCherry: fluorescent reporter protein, Cas9 protein: CRISPR endonuclease, kanR: kanamycin resistance, oriT: origin of transfer (for integration), and *smal*: representative kill gene (restriction endonuclease).

[00103] FIG. 12A-12C shows nucleotide sequence (SEQ ID NO: 131) of pIMAY Integrative Plasmid. (accession number JQ62198).

[00104] FIG. 13A shows activity of promoter candidates *isdA*, *isdB*, *hlgA2*, *hrtAB*, *isdG*, *sbnE*, *lrgA*, *lrgB*, *fhuA*, *fhuB*, *ear*, *hly*, *splF*, *splD*, *dps*, and SAUSA300_2617 at 1 min, 15 min and 45 min in serum and fold changes in gene expression vs. media by qPCR.

[00105] FIG. 13B shows activity of promoter candidates *isdA*, *isdB*, *hlgA2*, *hrtAB*, *isdG*, *sbnE*, *lrgA*, *lrgB*, *fhuA*, *fhuB*, *ear*, *hly*, *splF*, *splD*, *dps*, and SAUSA300_2617 at 1 min, 15 min and 45 min in blood and fold changes in gene expression vs. media by qPCR.

[00106] FIG. 14 shows inducible inhibition of cell growth of synthetic microorganism pTK1 cells comprising a cell death toxin gene (*sprA1*) behind a cadmium promoter on a pCN51 plasmid (pTK1) which had been transformed into *Staphylococcus aureus* RN4220 cells. OD (630 nm) read at 2 hrs post induction. Wild-type 4220 cells showed good cell growth both in the absence of cadmium and in the presence of 500 nM and 1 uM cadmium. pTK1-1 and pTK1-2 cells showed good growth in the absence of cadmium, but cell growth was significantly inhibited in presence of 500 nM and 1 uM cadmium at 2 hours post induction.

[00107] FIG. 15A shows a plasmid map of p174 (pRAB11_Ptet-*sprA1*) zoomed view of the region of the plasmid containing the Ptet-*sprA* cassette.

[00108] FIG. 15B shows the p174 (pRAB11_Ptet-*sprA1*) whole plasmid in its native circular form.

[00109] FIG. 15C shows photographs of plate dilutions at 6 hours synthetic microorganism *Staphylococcus aureus* 502a p174 cells comprising a cell death toxin gene (*sprA1*) behind an anhydrotetracycline promoter on a pRAB11-2 plasmid (p174) which had been transformed into *Staphylococcus aureus* 502a cells. The p174 plasmid containing a deleted *sprA1* antisense (Das). Plate dilutions at 10e-5 are shown after 6 hours of induction for uninduced (left) and induced (right) 502a p174 (tet-*sprA1*Das) cells on BHI chlor10. The plate on the left (Uninduced) was uncountable at 10e-5 but at 10e-6 counted ~ 720 colonies. The induced plate on the right at 10e-5 produced 16 colonies. The survival percentage of induced cells at 6 hours post induction was 0.22%.

[00110] FIG. 16 shows cell growth pre- and post-induction of four synthetic strains derived from *Staphylococcus aureus* 502a having a plasmid based inducible expression system comprising four different cell death gene candidates *sprA1*, 187-lysK, Holin, and *sprG*. The candidate cell death genes had been cloned behind an tetracycline inducible

promoter on pRAB11 plasmids and transformed into *Staphylococcus aureus* 502a cells. Calculated OD600 readings were taken at T=0, 30, 60, 120, and 240 min after induction of AtC induced (+) strains illustrated by dashed lines (- - - - -) and uninduced (-) strains indicated by solid lines (—) for BP_068 (502a pRAB11-Ptet-sprA1), BP_069 (502a pRAB11-Ptet-187lysK), BP_070 (502a pRAB11-Ptet-holin), and BP_071 (502a pRAB11-Ptet-sprG1) and compared to BP_001 (502a wt) in BHI media. Each of the induced (+) strains BP_068 (sprA1), BP_069 (187lysK) and BP_070 (holin) exhibited both (i) good cell growth pre-induction and (ii) significant inhibition of cell growth post-induction. BP_068 (+) exhibited the best inhibition of cell growth at each time point T=30, T=60, T=60, T=120 and T=240 min post-induction, so the sprA1 gene was selected for initial further development of a kill switch in *Staphylococcus aureus* 502a.

[00111] FIG. 17 shows a bar graph showing difference in the colony forming units(cfu)/mL between T=0 (gray) and 240 min(black) of un-induced (-) and anhydrotetracycline induced (+) strains BP_068 (502a pRAB11-Ptet-sprA1), BP_069 (502a pRAB11-Ptet-187lysK), BP_070 (502a pRAB11-Ptet-holin), and BP_071 (502a pRAB11-Ptet-sprG1) compared to BP_001 (502a wt) in BHI media. Each of the induced (+) strains BP_068 (sprA1), BP_069 (187lysK) and BP_070 (holin) exhibited both (i) good cell growth pre-induction and (ii) significant inhibition of cell growth post-induction. BP_068 exhibited the best inhibition of cell growth 240 min post-induction, so the sprA1 gene was selected for initial further development of a kill switch in *Staphylococcus aureus* 502a.

[00112] FIG. 18 shows GFP expression fold change of induced (+) and uninduced (-) subcultures of *Staphylococcus aureus* strains BP_001, BP_055 and BP_076.

[00113] FIG. 19 shows a map of the genome for Strain BP_076 (SA 502a, ΔsprA1::Ptet-GFP).

[00114] FIG. 20 shows a map of plasmid constructed for making genomic integration in *Staphylococcus aureus*.

[00115] FIG. 21 shows a map of PsbnA-sprA1 kill switch in *Staphylococcus aureus* 502a genome. Serum and blood responsive promoter PisdB is operably linked to sprA1 toxin cell death gene.

[00116] FIG. 22 shows a map of a kill switch construction using serum and blood responsive promoter PisdB operably linked to sprA1 toxin cell death gene and an

expression clamp comprising a second promoter *clfB* operably linked to *sprA* AS to prevent leaky expression of the toxin in the absence of blood or serum. The kill switch is incorporated to the *Staphylococcus aureus* 502a genome.

[00117] FIG. 23 shows a growth curve of three strains when exposed to human serum compared to TSB: 502a – *Staphylococcus aureus* wild type, *Staphylococcus aureus* BP_011 – 502a Δ sprA1-sprA1(AS), and *Staphylococcus aureus* BP_084 – 502a Δ PsprA::PsbA in which the kill switch is integrated to the genome of *Staphylococcus aureus* 502a. The dashed lines represent the strains grown in serum, and the solid lines represent the strains grown in TSB. After 180 minutes, the strain BP_084 with the integrated kill switch shows a growth curve that is significantly reduced compared to the wild type in serum and the kill switch in complex media. After 3 hours of exposure to human serum, the *Staphylococcus aureus* BP_084 (502a Δ PsprA::PsbA) cells exhibited 98.84% measurable average cell death compared to the same BP_084 cells in TSB.

DETAILED DESCRIPTION OF THE INVENTION

[00118] The present disclosure relies upon a principle known as "bacterial replacement", or "niche exclusion", where one microorganism replaces and excludes another. In the field of ecology, competitive exclusion, or Gause's Law, states that two species that compete for the exact same resources cannot stably coexist. This is due to the fact that one of the competitors will possess some slight advantage over the other leading to extinction of the lesser competitor in the long run. In higher order organisms, this often leads to the adaptation of the lesser competitor to a slightly different ecologic niche.

[00119] Methods and compositions for durably managing the microbiome of a subject are provided. In embodiments, the microbiome is a dermal and/or mucosal microbiome (Exobiome). While methods to treat infection by a pathogenic microorganism exist, methods to prevent recurrence are effectively nonexistent.

[00120] **Infectious Agent - *Staphylococcus aureus* (MSSA and MRSA)**

[00121] Classified since the early twentieth century as among the deadliest of all disease-causing organisms, each year around 500,000 patients in hospitals of the United States contract a staphylococcal infection, chiefly by *Staphylococcus aureus*. Up to 50,000 deaths each year in the USA are linked with *Staphylococcus aureus* infections.

Staphylococcus aureus exists on the skin or inside the nostrils of 40-44% of healthy people. *Staphylococcus aureus* is also sometimes found in the mouth, gastrointestinal, genitourinary, and upper respiratory tracts. Some studies indicate even higher colonization prevalence. For example, Eriksen et al maintain that there is a higher percentage of transient or intermittent carriers that increase the prevalence number; sometimes to greater than 75%.

[00122] *Staphylococcus aureus* 502a WT BioPlx-01WT® and Other Replacement and Blocking Strains

[00123] A *Staphylococcus aureus* 502a WT strain called BioPlx-01WT® is employed in example 1 and is a natural “wild-type” organism known to be relatively non-infectious, and which has no known side effects. It has been shown in BioPlx clinical studies to be highly effective in this intended application (occupying and blocking the required microbiomic niche to prevent the recurrence of MRSA).

[00124] The present methods prevent infection by durably replacing the (typically virulent and antibiotic-resistant) colonizing undesirable *Staphylococcus aureus* strain with a “blocking” organism – in this study the BioPlx01-WT *Staphylococcus aureus* 502a WT strain. This phenomenon is expected to be applied in a similar manner for any other pathogen replacement organism developed by BioPlx.

[00125] Other replacement strains such as synthetic strains are provided herein that are fully able to colonize the properly prepared skin and mucosal surfaces, and to occupy the ecologic niche used by this bacterial species, thereby blocking other variants from recolonizing that niche.

[00126] There are a very large number of *Staphylococcus aureus* variants (10,000+ genomes as of 9/2017), as well as a wide range of genetic cassettes and virulence factors associated with this species.

[00127] Methicillin-resistant *Staphylococcus aureus* (MRSA) refers to a class of antibiotic resistant variants of this common human commensal and sometimes pathogenic bacteria. It varies from the wild-type strain (MSSA – Methicillin Sensitive *Staphylococcus aureus*) by its carriage of a *mecA* cassette that allows MRSA strains to produce an alternate penicillin binding protein (PBP2A) that renders them resistant to treatment with most beta lactam and many other first-line antibiotics.

[00128] Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Virulent Methicillin-Susceptible *Staphylococcus aureus* (vMSSA) are virulent, invasive variants of *Staphylococcus aureus* that colonize many humans, and which can further cause both superficial soft tissue and severe systemic infections. Colonization with MRSA or vMSSA is usually a required precursor to active Staph infection. Infection is caused by the bacteria colony on the skin or mucosal membranes, penetrating the outer immunological barrier and invading tissue or the blood stream through a wound, an incision, a needle puncture, or other break in the skin. This can lead to bacteremia and other systemic infections that have high mortality rates.

[00129] The present disclosure uses a generally passive strain of *Staphylococcus aureus* to replace and exclude MRSA or vMSSA from its usual place in the dermal/mucosal microbiome. The wild type interfering *Staphylococcus aureus* used by BioPlx is known to be poor at causing systemic disease, however, regardless of the level of variance or invasiveness virtually any microorganism can become an "accidental pathogen" through natural or accidental inoculation. This is particularly true in the case of *Staphylococcus aureus*.

[00130] The decolonization and BioPlx01 strain application methods developed by BioPlx allows the strains provided herein a massive numerical and positional competitive advantage. The consequences of this method provide a much longer effect of MRSA decolonization than a simple antiseptic destruction of the virulent MRSA strain. Early studies show a greater than 6 month total exclusionary effect of the BioPlx01 MRSA decolonization/recolonization process with the BioPlx product as opposed to prior literature demonstrating 45% recurrence of *Staphylococcus aureus* nasal colonization at 4 weeks and 60% at 12 weeks with the standard decolonization method alone.

[00131] Overview of Indication

[00132] *Staphylococcus aureus* infections are a severe problem in both hospitals and community health settings. Methicillin-resistant *Staphylococcus aureus* (MRSA) is genetically different from other strains of *Staphylococcus aureus*, with genetic elements conferring resistance to the antibiotic methicillin and other (usually beta-lactam) antibiotics typically used to treat *Staphylococcus aureus* infections. MRSA strains carry a *mecA* expression cassette that allows MRSA strains to produce an alternate penicillin

binding protein (PBP2A), and it's this mutation that confers resistance. Due to this resistance, MRSA is difficult to treat, making it a life-threatening problem in many cases. MRSA is frequently contracted in hospitals or other types of healthcare settings (Hospital Associated [HA]). These infections typically occur at the time of an invasive procedure such as surgery, intravenous catheterization, intubation, or artificial joint placement. Community-associated (CA) MRSA is typically spread by skin-to-skin contact, and the first symptoms tend to be large boils on the skin.

[00133] The BioPlx method using BioPlx strains is not a treatment for invasive MRSA disease, and therefore is not intentionally applied to a patient during the invasive disease state. The benefits of the BioPlx method can be demonstrated in a patient group that: 1) is at high risk for invasive disease, 2) has high morbidity and mortality from this increased risk to show significant clinical benefit, and has no other effective options for the prevention of invasive *Staphylococcus aureus* disease. These characteristics define the group of patients that the Centers for Disease Control have been tracking regarding the MRSA subset since 2005 who have already experienced invasive MRSA disease – 72,444 according to ABC surveillance data in 2014.

[00134] The surface of the human skin and mucosal layer where *Staphylococcus aureus* resides in the colonization state has a very different level of required nutrients as well as different environmental qualities than that inside the human body. It has been widely recognized that in order for bacteria to be successfully invasive, they must be able to adjust their needs and responses between the colonization and invasive states. This is accomplished by the bacterium sensing the changes between these environments and switching on or off certain gene cassettes allowing for the production of proteins more adapted to the new invasive state.

[00135] The BioPlx method, and specifically BioPlx01 strains, take advantage of this requirement by rearranging molecular instructions leading to the death of the organism in the operons of one or more of these specific cassettes. This creates a “holding strain” of colonizing *Staphylococcus aureus* that is unable to cause disease in the patient to whom it is introduced, but also does not allow other circulating *Staphylococcus aureus* strains that may normally colonize the human population to colonize this patient. This occurs through the ecologic premise of competitive exclusion.

[00136] The current “Standard of Care” for patients colonized with MRSA is not uniform. There are no guidelines as to the management of staphylococcal colonization in patients that are at high risk of recurrent disease. The IDSA Clinical Practice Guidelines for the Treatment of MRSA Infections in Adults and Children in 2011 provide only C-III level (the lowest - no data, expert opinion) support for decolonization procedures in patients with recurrent community-acquired skin and soft tissue infections and make no mention of the role of decolonization in the prevention of invasive MRSA disease. Some hospitals have pursued a broad screening and isolation program for all admitted patients to their institution, but this has not been shown to be effective owing to (including) poor durability of effect and lower baseline risk of the average hospitalized patient (i.e. UC Irvine MRSA outbreak.) Other hospitals therefore have reduced their attention to patients admitted to the ICU and cardiothoracic surgery cases only. This strategy has been shown to reduce MRSA clinical isolates as well as bloodstream infection from any pathogen. However, these are short term situational strategies designed to reduce risk of MRSA infection over a near time frame.

[00137] MRSA disease and colonization is a complicated epidemiologic problem for both the United States and the rest of the world. The manifestations of MRSA are broad from asymptomatic colonization to invasive disease states conferring high mortality and cost to the system. It is clear that the MRSA patients that have experienced invasive disease is medically distinct. They have a higher mortality than any other MRSA subpopulation. They have a higher treatment failure rate. They have a much higher risk for another invasive MRSA incident than any other group of patients. This makes this group an appropriate orphan group toward which the BioPlx method should be directed, and which would benefit from its use.

[00138] It can be concluded that decolonization is largely ineffective in durably clearing MRSA colonization, and leads to a high rate of recurrence. We have found that only decolonization in conjunction with active recolonization provides long term conversion from one organism (variant) to another.

[00139] **Recurrent Invasive MRSA as a Clinically Distinct Disease**

[00140] Another indication is “prevention of recurrent invasive MRSA.” Patients who have already experienced an episode of invasive MRSA infection have a greatly increased susceptibility to a subsequent invasive MRSA infection. The BioPlx

technology provided herein works by occupying the niche in the microbiome that would normally have the potential to be occupied by a virulent form of MRSA.

[00141] Invasive MRSA-caused Systemic Infection:

[00142] SA, including the variant MRSA, can exist in harmless coexistence on the surface of the skin and mucous membranes of at least 40% of all humanity, so the bacterium itself is not descriptive of disease; rather, its clinical presentation is definitional.

[00143] The whole of national and international authorities that define and monitor this condition concur that invasive MRSA infection is a separate and distinct disease from other conditions caused by this bacterium.

[00144] Simple colonization with any type of *Staphylococcus aureus* should not be considered a disease state. In fact, those humans with nutritional and environmental characteristics of their skin and mucosal biomes that are hospitable to *Staphylococcus aureus* must have some such niche occupant as part of their microbial flora to achieve a stable balanced “resting state” of their biome. The goal of any method would be to durably replace a MRSA strain on an at-risk patient with the product strain – in this case an antibiotic sensitive *Staphylococcus aureus* modified to be unable to survive within the human body in the invasive state.

[00145] To create invasive infectious disease, MRSA must abandon its passive commensal status, and breach the dermal/mucosal barrier, entering into the subdermal interstitial (interstitial fluid) or circulatory (blood, serum, plasma) areas. This “state change” initiates a new disease state, with new organism behaviors and relationships to the host.

[00146] *Staphylococcus aureus* bacteremia (SAB) is an important instance of this type of infection with an incidence rate ranging from 20 to 50 cases/100,000 population per year (ranging from 64,600 to 161,500 cases per year). Between 10% and 30% of these patients will die from SAB. Invasive systemic MRSA bacteremia has a mortality rate of around 20%. Comparatively, this accounts for a greater number of deaths than for AIDS, tuberculosis, and viral hepatitis combined.

[00147] The latest report for which there is a CDC-US national case estimate for invasive MRSA disease (2014) is 72,444 cases. The number of patients with this disease is less than 200,000 per annum, and it may permit an orphan drug designation. MRSA

can impact patients at three distinct levels: 1) colonization , 2) superficial infection – skin and soft tissue, and 3) systemic invasive infection.

[00148] 1) Colonization. *Staphylococcus aureus* is a normal commensal organism permanently colonizing around one third of the human population, with transient colonization occurring in about one additional third of the population. MRSA variants of this organism occupy organism the microbiome niche, and have colonized approximately 2% of the population in the US (with a high degree of variability depending on location and occupation). MRSA colonization creates a standing reservoir of potentially infectious organisms located directly on the outer layer of our immune/defense system, and this poses an ongoing risk to the patient.

[00149] 2) Superficial infection – skin and soft tissue infection. Skin-associated MRSA or skin and soft tissue infection is the most common of the two major disease state categories. It typically starts as a swollen, pus or fluid filled, boil that can be painful and warm to the touch, and at times accompanied by a fever. If left untreated, these boils can turn into abscesses that require surgical intervention for draining. For MRSA that's confined to the skin, surgical draining of abscesses may be the only necessary treatment, and antibiotics are not indicated. Skin and soft tissue infections are treated by surgically draining the boil and only administering antibiotics when deemed absolutely necessary.

[00150] 3) Systemic invasive infection. MRSA bacteremia (invasive MRSA) is a systemic MRSA infection that is defined as the presence of MRSA in typically sterile sites, including the bloodstream, cerebrospinal fluid, joint fluid, bone, lower respiratory tract, and other body fluids. MRSA bacteremia has a far worse prognosis compared to MRSA infections confined to the skin, with 20% of cases resulting in death. The difference in prognosis, location of the infection, and clinical symptoms of the condition make it clinically distinct from skin and soft tissue infection MRSA infections. MRSA bacteremia causes multiple complications not seen in skin and soft tissue infections, including infective endocarditis, septic arthritis, and osteomyelitis. For invasive MRSA, daptomycin and vancomycin are recommended treatments in the U.S. Vancomycin has a relatively slow onset and poorly penetrates some tissues. Daptomycin has been shown to be effective, but treatment-emergent nonsusceptibility is an issue, in addition to the issue of vancomycin encouraging daptomycin resistance in MRSA. The difference in clinical symptoms as well as treatment methods for invasive MRSA provides clear

evidence for invasive MRSA as a clinically distinct condition from MRSA Skin and soft tissue infections.

[00151] The BioPlx technology works by preventing the recurrence of an invasive MRSA infection in those who have been colonized (including those that have already experienced an invasive MRSA infection) and who have undergone a decolonization procedure. As a decolonization/recolonization microbiotic method, the BioPlx technology would not be administered to “treat” a patient while they had a systemic MRSA infection. It would be applied subsequent to the clearance of a systemic MRSA infection (and a full body decolonization).

[00152] It is an established principle of medical nomenclature that a disease or condition is not simply synonymous with the causative agent. In the present case, MRSA-mediated systemic bacteremia (or other designations of invasive systemic disease) is unambiguously distinct from the other superficial skin and mucosal conditions that may be caused by, or associated with, MRSA, or by other *Staphylococcus aureus* strains. Invasive systemic MRSA-mediated disease has a clearly distinct diagnosis, pathology, treatment, and prognosis profile.

[00153] It’s important to note that, based on the mechanism of action of BioPlx01 strains, patients are prevented from subsequent systemic MRSA infection, as opposed to treatment of invasive MRSA infection per se. So, “prevention of recurrent systemic MRSA infection” would be the most accurate description of the indication for BioPlx01 strains.

[00154] The target population of patients that have had invasive MRSA Infection, have been successfully cleared of the organism (typically through standard antibiotic intervention (e.g. Vancomycin), and yet have a high risk (rate) of MRSA recolonization, recurrence and the associated elevated risk of MRSA systemic reinfection.

[00155] **International and US Recognition of the Disease Designation:**

[00156] A clear definition of this disease is put forth by the Centers for Disease Control and Prevention (CDC) as it has been actively monitoring this condition in the United States since 2005. The agency performs this monitoring utilizing the Active Bacterial Core surveillance system via the Emerging Infections Program (EIP). A case in this context is defined by the isolation of MRSA from a normally sterile body site. Normally sterile sites included blood, cerebrospinal fluid, pleural fluid, pericardial fluid,

peritoneal fluid, joint/ synovial fluid, bone, internal body site (lymph node, brain, heart, liver, spleen, vitreous fluid, kidney, pancreas, or ovary), or other normally sterile sites.

[00157] The CDC also created the National Healthcare Safety Network (NHSN) as a tracking system for more than 16,000 US healthcare facilities to provide data to guide prevention efforts. The Center for Medicare Services (CMS) and other payers use this data to determine financial incentives to healthcare facilities for performance. The system tracks MRSA bloodstream infections as a marker for invasive disease for epidemiologic purposes.

[00158] The MRSA mediated invasive disease state is also codified in the ICD9 and ICD10 system by a grouping of conditions each with their own numeric code specific for the causative agent MRSA. For example, sepsis due to MRSA is coded A41.02, pneumonia due to MRSA is coded J15.212. This further exemplifies the differential characterization that invasive MRSA disease is given in juxtaposition to superficial skin and soft tissue disease due to the same agent - code L03.114 (left upper limb example) with the follow code of B95.6 MRSA as the cause of disease classified elsewhere, which is attached to a variety of other infection codes to indicate MRSA as the cause of the disease condition.

[00159] The European Center for Disease Control (ECDC), a branch of the EU also surveilles invasive *Staphylococcus aureus* isolates by similar definition to the NHSN and tracks methicillin-resistance percentages but the reporting requirements do not produce an EU estimate of total annual cases.

[00160] Differentially, unlike systemic conditions, simple MRSA colonization is not itself typically regarded as a disease. Colonization however is considered a precondition for most invasive disease, as evidenced (for example) by studies that show that nasal *Staphylococcus aureus* isolates are usually identical to strains later causing clinical infection. This persistent colonization state reflects the ecological stability of this bacteria on skin and mucosal surfaces.

[00161] This colonization state is recorded in the ICD10 system, Z22.322, under the Z subheading which is reserved for factors influencing health status and contact with health services but not an illness or injury itself.

[00162] **The target orphan disease population:**

[00163] The orphan disease population targeted for the BioPlx non-recurrence method is the group of people previously invasively infected (systemic infection) with MRSA (a population known to be susceptible), and who continue to suffer ongoing recolonization with MRSA. CDC monitors all U.S. cases of invasive MRSA infection. Multiple researchers have described this medically distinct population – patients who have already suffered one defined episode of invasive MRSA infection. This group is at increased risk for life threatening invasive disease as a result of their demonstrated susceptibility and their continued colonization.

[00164] In some embodiments, a method is provided for preventing recolonization, or preventing recurrence of MRSA-caused systemic invasive bacteremia, comprising prevention of (or prevention of recurrence of) a prerequisite MRSA colonization by

- 1) decolonization of MRSA from mucosal and dermal microbiomes, and
- 2) recolonization of these microbiomes with a synthetic *Staphylococcus aureus* (e.g., a BioPlx01 strain). The method is effective, through the effect of bacterial interference, operating through niche dynamics within the target dermal/mucosal microbiome ecosystem, because the synthetic *Staphylococcus aureus* (e.g., a BioPlx01 strain) serves to occupy specific niches, and thus blocks/prevents MRSA recolonization (blocks recurrence). The efficacy of this method has been demonstrated clearly in proof of principle studies provided herein.

[00165] SA is present as part of the normal microbiome of more than 40% of the total human population. The MSSA colonization state is common. The MRSA variant is found on around 1-2% of the US population, but in certain areas or demographics this level can be considerably higher. It is thought that MRSA has the ability to colonize anyone within the *Staphylococcus aureus* susceptible population. *Staphylococcus aureus* lives most commonly on the surface of the skin and in the anterior nasal vestibules, but can also be found in smaller amounts in the deep oropharynx and gastrointestinal tract and in normal vaginal flora in some individuals.

[00166] In colonized individuals *Staphylococcus aureus* usually remains a non-invasive commensal bacterium simply occupying an ecologic niche and not causing disease. In a portion of those colonized however, this bacteria can cause disease either

opportunistically or as a result of the increased likelihood of invasion due to some particular variant characteristics.

[00167] Approximately 23% of persistent MRSA carriers developed a discrete MRSA infection within one year after identification as a carrier.

[00168] Many *Staphylococcus aureus* variants have acquired genetic cassettes coding for virulence protein products that allow such strains to more effectively invade through the epidermal or mucosal tissue layers, and subsequently initiating deep or systemic infection. In colonization or infection the presence of the *mecA* cassette limits the treatment options for these patients, and a number of studies have documented the increased mortality rate associated with MRSA when compared to MSSA in bacteremia, endovascular infection and pneumonia.

[00169] It is not possible to predetermine whether an individual who is colonized with MRSA will eventually progress to invasive disease or not, so it is particularly important to identify and treat the entire population of patients who have a well-documented increased risk for invasive MRSA disease.

[00170] **MRSA-mediated invasive disease statistics:**

[00171] MRSA was identified by British scientists in 1961 and the first American clinical case was documented in 1968. For the next 25 years, MRSA was regarded largely as an endemic hospital-based problem that was increasing in incidence, however starting in the mid to late 1990s, an increase of incidence of community-associated MRSA was seen mostly manifesting in superficial skin and soft tissue infections. Of greatest concern to the medical community has been the increase in invasive infections caused by MRSA. The increasing trend in incidence of invasive MRSA disease was seen throughout the 1990s and peaked in 2005.

[00172] The CDC tracks the incidence of invasive MRSA disease through the NHSN and the Emerging Infections Program – Active Bacterial Core surveillance system also starting in 2005. As compared to 2005, 2015 data shows that the overall incidence for invasive MRSA disease has decreased almost 50% from an incidence rate of 37.56 to 18.8. Expensive and laborious infection control interventions enacted in hospitals in response to this public health crisis has been given much of the credit for the decreased incidence, as the majority of the gain was seen in health care associated cases as opposed to community associated ones. Despite the gains that have been made over the past

decade, invasive MRSA infections continue to be a prioritized public health issue. These infections can be very difficult to treat and treatment failure has been shown in nearly 25% of patients on proper therapy. Predicting which health care experienced patients are at risk for invasive MRSA is a challenging problem. Risk factors such as MRSA colonization, the presence of chronic open wounds and the presence of invasive devices have been elucidated.

[00173] The presence of these characteristics alone do not predict which patient will ultimately display invasive disease. However, one of the most predictive risk factors for a patient getting an invasive MRSA infection is having had a previous invasive MRSA infection. In the 2004-2005 data from the Active Bacterial Core Surveillance (ABCs) it was noted that almost 13% of their invasive cases went on to develop a second invasive MRSA infection during the 18 months of retrospective data evaluation. Another look at the EIP-ABC data in the calendar year 2011 found that 8% of these patients had more than one invasive MRSA infection separated by at least 30 days. The longer term risk of recurrent invasive MRSA infection is surely greater still as these estimates will miss earlier infections in these patients prior to the study time period and later ones that occur after the end date. Since Huang and Platt (2003) showed that 29% of hospitalized patients with known MRSA colonization or infection went on to develop a second MRSA infection (often severe) within an 18 month follow up, targeting this group to prevent recurrence of the invasive disease state could prevent approximately 17,500 subsequent invasive MRSA infections (using the most recent CDC data).

[00174] Invasive MRSA and skin and soft tissue infection from MRSA are both caused by the same pathogen. However, orphan designations are awarded based on the dyad of **drug** and **disease**. MRSA is a pathogen, and not a disease state. However, it can cause infection, and it's these different types of infectious disease that are being treated. Invasive MRSA comes with a far more severe prognosis as well as different clinical manifestations from MRSA confined to the skin or simply being colonized with MRSA. About 40% of the U.S. population is colonized with *Staphylococcus aureus*, typically found in the nose or on the skin. Generally, there are no signs of infection that would be considered "a disease state." However, systemic MRSA infection will manifest as high grade fever, chills, dizziness, chest pain, swelling of the affected area, headache, rash, cough, and other systemic symptoms. These two conditions are treated differently, where skin and soft tissue infections are typically treated by incising and draining the boils

commonly associated with skin and soft tissue infections. Antibiotics and decolonization are only employed if there are signs of systemic or severe disease that has spread to multiple sites.

[00175] Invasive MRSA has an incidence rate of 20 to 50 cases/100,000 people per year.^{6a} With a current U.S. population of 326,199,002 (accessed on November 2, 2017 from www.census.gov/popclock), this means there are 163,100 cases of invasive MRSA infection in the U.S. per year conservatively, falling below the 200,000 patient criteria for FDA orphan designation. We searched for other sources of reported prevalence to confirm that we had calculated the most conservative estimate of this patient population. Hassoun et. al reported an incidence of 72,444 cases of invasive MRSA in the U.S. in 2014, which had decreased from 111,261 in 2005.^{7a} Based on this, and assuming that the population will continue to decrease, we can assume that a prevalence of 163,029 patients with invasive MRSA in the U.S. in 2017 is a very conservative estimate. According to the CDC, there were more than 80,000 invasive MRSA infections and 11,285 related deaths in 2011.

[00176] To address this problem the present inventors have developed BioPlx01 strains, molecularly-altered strains of *Staphylococcus aureus* that are unable to cause disease but can reside in the microbiome niche that MRSA could take hold in. The lack of invasiveness of BioPlx01 strains is made possible by operons that are turned on upon contact with blood or plasma, triggering the death of the organism. A patient who has tested positive for MRSA and is experiencing systemic symptoms will undergo a full body decolonization before the BioPlx01 strain is administered, allowing it to occupy the niche that MRSA would have previously occupied in that patient's microbiome. By preventing virulent strains of MRSA from occupying the niche, these virulent strains cannot colonize, and subsequently invade sterile tissue sites. BioPlx01 strain is able to prevent recurrent systemic MRSA infections.

[00177] In one embodiment, a method for treatment of *Staphylococcus aureus* lung infections in patients with cystic fibrosis is provided.

[00178] In one embodiment, a method for treatment of Invasive Bacteremia is provided. Using the criteria adopted by CDC (Centers for Disease Control and Prevention), Invasive Bacteremia is indicated by the isolation of bacteria from a normally sterile body site. These may include blood, CSF, joint fluid, bone samples, lower

respiratory tract samples and other sterile body fluids. This condition is related to, but is clearly distinguished from, simple bacterial colonization and bacteria mediated skin and soft tissue infection. It is accepted that the colonization state is a prerequisite for invasive disease in the vast majority of cases.

[00179] MRSA and v-MSSA Mediated Invasive (Systemic) Bacterial Infection

[00180] Mediated by *Staphylococcus aureus*, MRSA Invasive Bacterial Infection may also be referred to commonly or in the literature as: MRSA bacteremia or sepsis, Systemic MRSA infection, MRSA bloodstream infections, invasive MRSA infection. Specific MRSA induced systemic conditions range from osteomyelitis, septic arthritis, pneumonia, endocarditis, bacteremia, toxic shock syndrome, to septic shock. The development of a method to prevent or reduce the recurrence of invasive MRSA disease in high-risk populations, through the mechanism of durably interfering with colonization of undesirable strains, would be a significant advance in the prevention of conditions typically required for invasive MRSA infection, and would reduce the likelihood of these patients suffering a subsequent invasive MRSA infection.

[00181] One objective of the present disclosure is to evaluate the BioPlx-01 WT material's ability to prevent the recurrence of MRSA in active healthy adult medical workers. This population is particularly at-risk for MRSA infection and has amongst the highest rates of MRSA colonization of any demographic. Successfully demonstrating a protective effect for this group would validate BioPlx-01 WT's efficacy in being able to prevent MRSA recurrence amongst effectively all those who are at risk.

[00182] "Recurrence" simply means "the bug comes back". Recurrence is of central importance to both disease evolution and control. With recurrence, the pathogen comes back again and again, and each time it goes through a survival cycle it "learns" to be more and more resistant to the antibiotics it has seen. Without this recurrence, once the pathogen is gone, it would stay gone, and that would be that. If there were no recurrence, there would be no pressure to evolve toward antibiotic resistance.

[00183] In various embodiments, the subject may be colonized with one or more pathogenic microorganisms. In certain embodiments, the undesirable microorganism is a drug-resistant pathogenic microorganism. The drug-resistant pathogenic microorganism may be selected from a *Neisseria gonorrhoeae*, fluconazole-resistant

Candida, MRSA, drug-resistant *Streptococcus pneumoniae*, drug-resistant Tuberculosis, vancomycin-resistant *Staphylococcus aureus*, erythromycin-resistant Group A Streptococcus, and clindamycin-resistant Group B Streptococcus. https://www.cdc.gov/drugresistance/biggest_threats.html.

[00184] In one embodiment, the undesirable microorganism may be a drug-resistant pathogenic *Staphylococcus aureus*.

[00185] Staphylococci are the most abundant skin-colonizing bacterial genus and the most important causes of nosocomial infections and community-associated skin infections. The species *Staphylococcus aureus* may cause fulminant infection, while infections by other staphylococcal species are mostly subacute. Colonization is usually a prerequisite for infection. Otto 2010, Expert Rev Dermatol 2010 Apr; 5(2):183-195. However, not all invasive *Staphylococcus aureus* infections are preceded by detected colonization with identical strain. The non-correlative fraction may be explained either by the “direct inoculation” or “direct wound seeding” theory such as an intraoperative event from a second carrier, or incomplete detection of all of these patient’s *Staphylococcus aureus* strains in colonization or colonization with the invasive strain in the time since the initial colonization surveillance.

[00186] SA is a common human commensal organism that is present (colonizes), typically without symptoms, in 30 to 50% of the (US) population. The asymptomatic carriage of *Staphylococcus aureus* by humans is the primary natural reservoir, although domestic animals, livestock, and fomites may serve as adjunctive reservoirs.

[00187] There are many different strains of *Staphylococcus aureus*, many of which can also act as serious pathogens. Symptoms of *Staphylococcus aureus* infections can be diverse, ranging from none, to minor Skin and soft tissue infections, to invasive life-threatening systemic disease such as endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, sepsis, toxic shock and endocarditis. The anterior nasal mucosa has traditionally been thought to be the most frequent site for the detection of colonization of healthy carriers with *Staphylococcus aureus*. Several sites may become asymptotically colonized including the nares, throat, axilla, perineum, inguinal region, and rectum.

[00188] MRSA isolates were once confined largely to hospitals, other health care environments, and patients frequenting these facilities. Since the mid-1990s, however,

there has been an explosion in the number of MRSA infections reported in populations lacking risk factors for exposure to the health care system. This increase in the incidence of MRSA infection has been associated with the recognition of new MRSA clones known as community-associated MRSA (CA-MRSA). CA-MRSA strains differ from the older, health care-associated MRSA strains; they infect a different group of patients, they cause different clinical syndromes, they differ in antimicrobial susceptibility patterns, they spread rapidly among healthy people in the community, and they frequently cause infections in health care environments as well. David, Michael et al., 2010, Clin Microbiol Rev 23(3): 616-687.

[00189] Why recurrent CA-MRSA Skin and soft tissue infections are common is not known. The mechanism by which recurrence occurs is unclear. Possibilities include reinfection from persistent asymptomatic CA-MRSA carriage or after acquisition from environmental MRSA or after new MRSA acquisition from close human or animal contact. Skin and soft tissue infections caused by MSSA also recur but less frequently than those caused by MRSA.

[00190] Under constant antibiotic pressure, many *Staphylococcus aureus* variants have developed antibiotic resistance. Today penicillin resistance in *Staphylococcus aureus* is virtually universal, and general beta-lactam and related multi-antibiotic (methicillin) resistance is now widespread, creating a significant new class of antibiotic-resistant "super-bugs".

[00191] The pathogenic *Staphylococcus aureus* may be a drug-resistant *Staphylococcus aureus*, such as MRSA, or a vancomycin-resistant strain, such as VISA or VRSA. Alternatively, the pathogenic *Staphylococcus aureus* may be a virulent methicillin-susceptible *Staphylococcus aureus* (v-MSSA). v-MSSA is a high-virulence cause of life-threatening invasive infections. MRSA and v-MSSA are epidemic, and have a high human cost.

[00192] MRSA has become a serious public health problem in hospitals, clinics, prisons, barracks, and even in gyms and health clubs around the world. MRSA is a common cause of hospital-acquired infections (500k US patients/year), and increasingly, of community acquired infections which can be serious. For systemically invasive disease – 20% of cases result in death. MRSA is one of the most significant of the new antibiotic-resistant "super-bugs". While methods to treat *Staphylococcus aureus*

infection exist, methods to prevent recurrence are effectively nonexistent. Recurrence of MRSA skin infections is found in 31% to 45% of subjects.

[00193] One effort to prevent recurrence includes decolonization. The first (and currently only) widely practiced step for preventing recurrence is decolonization. Unfortunately, simple decolonization is poor at preventing recurrence. Doctors can initially treat the microbial colonization or infection-for example MRSA or v-MSSA colonization/infection- with topical chemicals (e.g. chlorhexidine) or antibiotics. In many cases treatment with antibiotics may “clinically” eliminate the disease. Antiseptics and astringents may be used for decolonization (i.e., suppression) including tea tree oil and chlorhexidine. Antibiotics used for suppression include topical antibiotics for nasal decolonization such as mupirocin. Systemic antibiotics most frequently used for MRSA include vancomycin, first generation antibiotics such as cefazolin, cephalothin, or cephalexin; and new generation antibiotics such as linezolid or daptomycin. In less serious MRSA cases, clindamycin or lincomycin may be employed. Nonetheless, with this decolonization alone the MRSA and v-MSSA pathogens typically recur-or grow back -nearly 1/2 of the time. This level of performance has naturally led to skepticism as to the efficacy of simple decolonization in preventing recurrence.

[00194] Clinicians often prescribe topical, intranasal, or systemic antimicrobial agents to patients with recurrent skin infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in an effort to eradicate the staphylococcal carrier state. Some agents can temporarily interrupt staphylococcal carriage, but none has been proved effective for prevention of skin infections caused by MRSA. Creech et al. *Infect Dis Clin North Am*. 2015 September ; 29(3): 429–464 .

[00195] In both the literature and in the hands of the present inventors, it has been found that the quality of decolonization is correlated to the recurrence rate observed, but simple decolonization rarely resulted in a durable, successful, outcome.

[00196] The present disclosure provides methods and compositions focused on preventing recurrence through the effective and durable modification of microbiome populations.

[00197] Methods for preventing or decreasing recurrence of a pathogenic microbial infection have been developed comprising suppressing a microbial infection or colonization.

[00198] A method to decrease recurrence of a pathogenic infection or decrease colonization of a undesirable microorganism in a subject is provided, comprising decolonizing the undesirable microorganism on at least one site in the subject to significantly reduce or eliminate the presence of the undesirable microorganism from the site; and replacing the undesirable microorganism by administering to the subject a synthetic second microorganism having the same genus and species as the undesirable microorganism.

[00199] The methods and compositions to prevent recurrence include replacement of the pathogenic microorganism by filling the biome niche occupied by the pathogen with a specially designed synthetic microorganism – or “good bug”. By occupying the same biome niche, the “good bug” crowds out the pathogen, preventing it from recolonizing, or moving into (or back into) its preferred ecological neighborhood. One way to ensure the same biome niche is filled is by designing a synthetic microorganism starting from the same genus and species as the pathogenic microorganism.

[00200] The methods and compositions to prevent recurrence include promoting or supporting the synthetic microorganism – the “good bug” – by re-establishing key nutritional, chemical, or commensal environments that further promote the preferred organism and inhibit recolonization by the pathogen. For example, a commensal cluster may provide further layered defense in preventing the pathogen from moving back into its old ecological niche – it may help prevent recurrence.

[00201] The BioPlx method is enabled by state of the art methods/technologies including microbiomics, systems & computational biology; environment interactions (clusters & signaling); proprietary organisms (selected & modified); and variant and strain substitution strategies.

[00202] Replacement microorganisms are provided herein including (1) “BioPlx01-WT® variant” – a *Staphylococcus aureus* 502a wild-type microorganism with an established history of non-virulence and passive colonization which has been isolated, verified, and prepared for field trials using this strain cluster as described in Example 1; (2) “BioPlx01-KO® engineered variant”, a synthetic *Staphylococcus aureus* strain that enhances safety by knocking out specific virulence genes; and (3) “BioPlx01-KS® engineered variant”, a synthetic *Staphylococcus aureus* strain that embeds a molecular programmed cell death trigger to prevent invasive virulence. In some

embodiments, the synthetic microorganism acts purely as a substitution for the pathogenic strain, without “new” infection or colonization.

[00203] An extensive proprietary library of fully characterized *Staphylococcus aureus* cultures (strains and variants) has been developed which is used for replacement organism sourcing; used for durability and competition analysis; used for Genotype/Phenotype comparative analysis; used for virulence genome/transcriptome clustering modeling; and used for signaling genome/transcriptome clustering modeling.

[00204] A Library of controlled commensal organisms is being developed for potential variant cluster co-administration with the BioPlx01-KS® variant.

[00205] Methods for Computational Microbiology are also being developed including Machine Learning; Modeling of complex dynamic microbiomic systems; Genome/Transcriptome/Proteome (Phenotypic) relationships; Virulence factor genetics and promoters; Modeling resilience and changes over time/condition; n-dimensional niche-forming relationships; and High dimensional cluster relationships.

[00206] Central to the present model anti-recurrence method is the principle of “non-co-colonization”, meaning that only one species, and one variant of that species, can occupy the relevant skin or mucosal biome ecological niche at any one time. Underlying this simple and testable phenomenon are a host of deeper generative principles that combine to shape the emerging science of Microbiomics. Although widely generalizable, discussion of non-co-colonization in this section refers specifically to *Staphylococcus aureus* colonizations.

[00207] Non-co-colonization

[00208] The principle of non-co-colonization (also known as “bacterial replacement”) states that only one variant/strain of one species can occupy any given niche within the biome at any given time.

[00209] The central empirical phenomenon of non-co-colonization represents an aggregate effect: the consequence of the interaction of a large number of forces that can be found operating in complex systems, and which are only today becoming well characterized and mathematized.

[00210] Bacterial niches within the human biome that are specific to the species level underlie the present technology. If there were no specificity to biologic niche

occupation, then intentional strain exchange would not be achievable, as would the experimentally demonstrated phenomenon of bacterial replacement.

[00211] Expectations for non-co-colonization are important for durability of the present method for prevention of recurrence of pathogenic colonization or infection. Variant-to-variant non-co-colonization has been demonstrated experimentally in the literature with strain/variant substitution (e.g., the *Staphylococcus aureus* 80/81 to 502a conversions of Shinefield et al., 1963) and has been confirmed in present clinical studies, as shown in Example 1.

[00212] Sustained species-to-species niche occupation is suspect because careful reading of the literature indicates that durability is low, and in vivo evidence is rare. A transient occupation may occur, but is not considered to be an important outcome, as we are only interested in durable outcomes.

[00213] Failure of durability in species-to-species substitution serves as evidence that specific niche-filling requires a “close variant” substitution. This is significant as only durable biomes can display the functional characteristics (such as resilience) required for an effective non-recurrence technology/product.

[00214] In the case of variant-to-variant replacement, such as that seen in the present disclosure with respect to MRSA anti-recurrence materials, no direct evidence from the literature has been identified as to whether the replacement requires a “biome disruptive event” (such as accidental or intentional decolonization by antimicrobials, antibiotics, etc.) or whether it can occur via a “slow competitive replacement” (one organism out competing another for resources, growth, etc.). However, overwhelmingly in human dermal biomes, only one strain colonizes a person “in toto”, indicating that slow competitive replacement occurs. Further, the 55% success rate of anti-MRSA decolonization methods show that “biome disruptive events” can also induce durable biome changes. Both of these phenomena are expressions of non-co-colonization.

[00215] Non-co-colonization occurs in nature, for example, in the vast majority of cases only one variant of *Staphylococcus aureus* is detected within a single biome (over 95% of cases, with the balance likely caused by “transient conditions”).

[00216] In specifying and evaluating non-co-colonization durability (efficacy) it is necessary to recognize three distinct scales of outcomes: (1) short-term – immediately

post recolonization, (2) early stable stage – after shedding excess organisms, and (3) long-term – after a stable “new” biome is established.

[00217] In the short-term – immediately post recolonization, the decolonized biome is dominated by organisms applied “in excess” during recolonization – generating a type of adventitious and transient binding (like spreading peanut butter). Testing within this period can only confirm that the biome application has occurred. Duration = a few days, with subsequent shedding of excess organisms.

[00218] In the early stable stage – after shedding excess organisms, the biome *per se* is reestablishing its equilibrium state, but ostensibly with the replacement organism rather than the pre-existing pathogen. Confidence in this outcome is primarily due to the overwhelmingly large ratio (probably millions to one) of new organisms to surviving post-decolonization pathogens. It is expected that this will become a stable colonization with a high level of durability. Testing at this period would confirm that MRSA or vMSSA has been eliminated, and replacement strain has been re-colonized. Duration = weeks to months.

[00219] In the long-term – after stable “new” biome established will demonstrate not only the organism's ability to occupy or “take” a niche, but its ability to “hold” that niche. In some embodiments, this stage is used to evaluate how competitive the replacement strain or synthetic microorganism is against the current generation of new biome invaders (such as USA300). This question refers to the “new” replacement organism’s ability to compete over time against a slow competitive replacement as well as by external forces that could be biome disruptive over time such as antibiotic or antiseptic exposures or frequent re-exposure to the pathogen – especially if the strains are differentially sensitive to this disruptor.

[00220] It is important to characterize the phenomenon of microorganism variant non-co-colonization, variant-versus-variant niche occupation, and the empirical evidence already developed that this phenomenon exists and is a strong force in the dermal biome ecosystem.

[00221] The law of “competitive exclusion” refers to the situation where only one organism dominates one niche.

[00222] One historical error in understanding this phenomenon is assuming this is a binary system, conceptually driven by either one or two variants. In fact, a large number

of different microorganisms, for example various *Staphylococcus aureus* strains may be environmentally present at any one time, and over time.

[00223] It may be concluded that without the phenomenon of non-co-colonization, virtually all “staph-capable” biomes would inherently be highly variable mixed heterologous “soups” of multiple variants. Various possibilities are shown in Table 1.

[00224] Table 1. *Staphylococcus aureus* (SA) niche compatibilities and expected outcomes

case	Niche compatibility	Competitive exclusion	Expected Outcome
1)	one <i>Staphylococcus aureus</i> niche	+	one variant dominates (except transitional)
2)	one <i>Staphylococcus aureus</i> niche	-	always large number of variants (soup)
3)	multi <i>Staphylococcus aureus</i> niches	+	any smaller # of variants = # of discrete niches
4)	multi <i>Staphylococcus aureus</i> niches	-	always large number of variants (soup)

[00225] In Table 1, cases 2 & 4 can be eliminated, because co-colonization occurs in under 5% (in literature), and even in these cases the vast majority of co-colonization instances observed involve only one other organism. Case 3 can be considered as possible in a low number of cases (less than 5%) potentially relating to incomplete or non-overlapping footprints of the niche vs replacement organism.

[00226] There is no direct evidence from the literature as to whether the observed replacement of one variant for another (e.g. acquisition of MRSA) is caused by a biome-disruptive event or from a slow competitive replacement. However, it is empirically clear that only one strain at a time tends to colonize any individual biome (in toto). Biogeographically distinct and distant sites within a given biome strongly tend to have the same variant, and this occurs without any observable total body decolonization and replacement process, indicating that a rule-driven competitive replacement process occurs. The observation of competitive replacement is another expression of the principle of non-co-colonization.

[00227] In hypothetical cases where the replacement variant does not fill the niche completely there may be a weak tendency to co-colonization. In these cases, a variant

cluster may be used to "fill the slots" with alternatives so that the co-colonization favors a synthetic replacement microorganism rather than the original pathogen. While this may involve the use of a different replacement microorganism, this is not recurrence – this is further blocking of recurrence.

[00228] Current evidence of non-co-colonization

[00229] One large study looked at the prevalence of co-colonization in 3,197 positive *Staphylococcus aureus* samples taken from healthy patients in Oxfordshire, England followed longitudinally for up to two years; the point prevalence of having multiple strains of *Staphylococcus aureus* in nares samples was 3.4 to 5.8%. Votintseva et al., 2014 J Clin Microbiol, 52 (4): 1192-1200. Of the *Staphylococcus aureus* carriers who submitted swabs nearly every two months for two years, 11% had transient co-carriage. The study used an effective spa typing protocol that allowed for a sensitive procedure for finding even low proportion co-colonization strains. The interpretation of this data set shows that *Staphylococcus aureus* colonization is a dynamic process with low prevalence of multiple *Staphylococcus aureus* strains vying for presence in the same niche over time. A simple calculation can establish that the observed results are not simply the independent occupation of a non-specific niche. In this instance, 1000 patients were screened and 360 were found to be *Staphylococcus aureus* positive. In a non-specific niche scenario, $.36 \times .36$, or 13%, (130 persons), would be expected to display co-colonization; however only 3.9% of the 360 carriers, (14 persons) at that primary point were in fact co-colonized, demonstrating the strain specificity of the microbiome niche for *Staphylococcus aureus*.

[00230] A small percentage of *Staphylococcus aureus* carriers may be transiently colonized with two different strains of *Staphylococcus aureus* at any incident time point. As discussed above, Votintseva et al, looked at all variants within MSSA and MRSA and reported point incidences of this phenomenon to be in the range of 3.4 – 5.8%. The paper looking only at mixtures of MRSA and MSSA (would only find species that differ at the *mecA* site) is predictably lower at 2.3%. If co-colonization was a stable state, mixtures of *Staphylococcus aureus* species would be expected in virtually all samples. This is not observed.

[00231] Another study looked at 680 patients presenting for any type of hospital admission. It was practice of the National Health Service at that time to screen all patients

being admitted for MRSA. Dall'Antonia, M. et al., 2005, J Hospital Infect 61, 62-67. During this evaluation the protocol was refined to discover MSSA, MRSA and co-colonized MRSA and MSSA patients. MSSA alone was found in 115 patients (16.9%), MRSA alone was found in 56 patients (8.2%) and co-colonization was discovered in 4 patients (0.58%), again supporting the view of a strain-exclusive niche in the microbiome for *Staphylococcus aureus*. It supports the concept that one *Staphylococcus aureus* strain can prevent the establishment of another. The results suggested a lower percentage of co-colonized carriers as would be predicted by the null hypothesis indicating that there is a significant protective effect against one *Staphylococcus aureus* strain colonization by a previous occupying resident *Staphylococcus aureus* strain. The statistical significance was $p < 0.01$. The protective effect of MSSA colonization against MRSA colonization was calculated to be 78% (CI: 29-99%).

[00232] A further study looked at non-concordant *Staphylococcus aureus* isolates in a population composed of HIV infected IV drug users in a methadone clinic. There were 121 baseline positive *Staphylococcus aureus* samples and 4 of these showed clear discordance among 3 colonies evaluated by PFGE. However, re-evaluation of these 4 samples showed that 2 of the 4 were concordant at second evaluation. No discordance was found after re-evaluating 18 samples first found to be concordant. Therefore 1.7 – 3.3% of this population was found to have co-colonization at a singular time point. Cespedes C. et al., J Infect Dis 2005; 191: 444–52.

[00233] Historical Evidence of decolonization/recolonization studies also show evidence of Non-Co-Colonization. This principle has been previously partially demonstrated during the 1960s and 1970s in the well-known 80/81 to 502a "bacterial interference" studies and clinical applications. Absence of co-colonization is shown in the early bacterial interference papers in the 1960s and 1970s, these papers also clearly demonstrate “competitive exclusion” in regulating co-colonization. Mixed cultures of both 80/81 as the resident strain and 502A as the donor strain were not observed, experimentally demonstrating non-co-colonization as a stable situation for the microbiome. (Shinefield et al., 1963; Shinefield et al., 1966; Shinefield et al., 1973; Aly et al., 1974; Boris et al., 1964; Light et al., 1967; Fine et al., 1967).

[00234] Without “non-equivalence” and “competitive exclusion”, microbiome niches would consistently be filled with multiple strains of the same species of bacteria. The isolation in nature of a pure strain culture of *Staphylococcus aureus* from the nares

would be a rare event if ever seen. The population dynamic in such a state would create a heterogeneous “soup” of many varieties of *Staphylococcus aureus*, as dictated by adventitious or random exposure from the environment. Any strain that the host has ever come in to contact with would have equal opportunity to colonize that space without competition or interference with any other strain variant (polyclonal colonization). The absence of this empirical result demonstrates “competitive exclusion”.

[00235] Yet, the exclusion principle is not so rigid that once a niche is occupied no other variant can usurp its position. These observations demonstrate an exclusion principle that is robust, but that allows external species to challenge an occupying species by briefly sharing that niche while the ultimate competition for dominance in that space is being enacted. On some occasions “new” strains overcome the previous resident strain and establish a new dominant resident strain. On other occasions, the interloper is rebuffed and the resident strain repels the attempt at replacement and reestablishes singular dominance. In both of these scenarios, the co-colonized state is transient and unstable; present at a low frequency.

[00236] Microbiomic Systems

[00237] Methods and compositions are provided to durably and safely prevent recurrence of a pathogenic microbial infection in a subject, comprising suppression of a pathogenic microorganism, replacement with a synthetic microorganism capable of occupying the same niche to durably exclude the pathogenic microorganism, and promotion of the synthetic microorganism for durable residence within the niche. This method is termed the BioPlx® method, as discussed above. In some embodiments, the subject is found to be colonized with the pathogenic microorganism prior to the suppression step.

[00238] In order to successfully work within the microbiome to promote the colonization of a desired organism in such a way as to produce a durable protective outcome requires that we know the “rules” of microbiomes: as discussed in greater detail in the sections following.

[00239] A non-co-colonization model has been developed to provide context and establish target product characteristics. The rationale for the present technology rests on the Microbiomic paradigm (biome/ecosystem/niche), and on the Microbiome having certain persistent and verifiable characteristics. The key discoverable metric rests on co-

colonization statistics in literature modified by specifics on decolonization, testing, and other relevant conditions, followed by direct observations from the clinical study of example 1.

[00240] The skin microbiome in the subject is an entity, a persistent identifiable thing. Over 10,000 different species of microorganisms make up the skin microbiome. The skin biome is an ecosystem which may be defined as a system, or group of interconnected elements, formed by the interaction of a community of organisms with their environment. The skin microbiome ecosystem has a “healthy”, or “normal” base state. The biome can be “healthy” or “sick” (dysbiosis), and can be invaded by pathogenic organisms – in other words the Microbiome can be invaded by a “Bad Bug” – such as MRSA – it can also become infected or contaminated by undesirable organisms or variants (dysbiosis). Dysbiosis is a term for a microbial imbalance or maladaptation on or inside the body, such as an impaired microbiota.

[00241] The skin microbiome has a structure created by a vast combinatorial web of relationships between the host and all of the components of the biome. The microbiome, or biome, is a dynamically structured complex system and is an "elastically resilient" ecosystem.

The skin microbiome has a dynamic but persistent structure – it is “resilient”, for example, even under conditions of massive cell death (e.g. washing, using ethanol, hand sanitizer, etc.) the biome regenerates in a similar form.

[00242] Resilience

[00243] The human microbiome has the quality of resilience meaning that mild perturbations tend to re-correct toward a previous established baseline of species mixture and concentration. However, members of each niche can be successfully challenged for their place in that stable mixture either as a result of an acute external disruptive event (i.e. an antimicrobial medication or an antiseptic application) or as a slow competitive replacement.

[00244] In ecology, resilience is the capacity of an ecosystem to respond to a perturbation or disturbance by resisting damage and recovering quickly. Resilience refers to ecosystem's stability and capability of tolerating disturbance and restoring itself.

[00245] In the literature, the main mathematical definitions of resilience are based on dynamical systems theory, and more specifically on attractors and attraction basins.

The human microbiome operates in many ways like a multi-basin complex system. It changes states or basins, but then resilience stabilizes that state. Martin, S. et al., 2011, in: Deffuant G., Gilbert N. (eds) Viability and Resilience of Complex Systems. Understanding Complex Systems. Springer, Berlin, Heidelberg, pp. 15-36.

[00246] The microbiome operates in many ways like a multi-attractor complex system - it can changes its states or basins, but then the resilience associated with that attractor stabilizes that state.

[00247] Ecological resilience is defined as the capacity of a system to absorb disturbance and reorganize while undergoing change so as to still retain essentially the same function, structure, identity and feedbacks. Mitra, C., et al., 2015, An integrative quantifier of multistability in complex systems based on ecological resilience, Nature, Scient. Rep., 5, 1–12.

[00248] The “competitive exclusion principle” provides that complete competitors cannot exist. The “axiom of inequality” states that no two things or processes in a real world are precisely equal. Hardin, 1960, Science, vol. 131, 1292- 1297, p. 1292. Based on Hardin's 'Axiom of Inequality' and the Competitive Exclusion Principle, long-term durability should only be achieved by close variant substitution, but would not likely be available with respect to species substitution. For example, MRSA and MSSA can co-colonize briefly – just like any other variants of *Staphylococcus aureus* can co-colonize in transient fashion. See Dall’Antonia, M. et al., 2005, J Hospital Infect 61, 62-67, disclosing a study of 680 patients presenting for any type of hospital admission and screened all patients being admitted for MRSA. During this evaluation the protocol was refined to discover MSSA, MRSA and co-colonized MRSA and MSSA patients. MSSA alone was found in 115 patients (16.9%), MRSA alone was found in 56 patients (8.2%) and co-colonization was discovered in 4 patients (0.58%), again supporting the view of a strain-exclusive niche in the microbiome for *Staphylococcus aureus*. It supports the concept that one *Staphylococcus aureus* strain can prevent the establishment of another.

[00249] Resilience may create recurrence – an observed natural phenomenon – as the existing (MRSA contaminated) biome tries to preserve itself.

[00250] However, resilience can also prevent MRSA recurrence – as exhibited by methods and compositions provided herein. By suppressing a pathogenic microorganism such as MRSA (“bad bug”) colonized in a subject, and replacing with a safe synthetic

microorganism (“good bug”) of the same species, it has been established that the “good bug” durably prevents recurrence of the “bad bug”(prevents MRSA re-invasion).

[00251] A historical example of resilience creating durable, persistent substitution is seen in *Staphylococcus aureus* carriers and replacement with strain 502a. Aly et al., 1974 J Infect Dis 129(6) pp. 720-724, studied bacterial interference in carriers of *Staphylococcus aureus*. The carriers were treated with antibiotics and antibacterial soaps and challenged with *Staphylococcus aureus* strain 502a. It was found that full decolonization was needed to get good colonization of 502a. Day 7 showed 100% take, but at day 23 the take was down to 60 to 80%. The persistence data was 73% at 23 weeks for well-decolonized subjects. Thus, long-term durability is only achieved by close variant substitution. Commensal microflora (normal microflora, indigenous microbiota) can help recolonization dynamics, but they do not fulfill close variant durability requirements. The inventors have designed a method for obtaining a “passive” version of an organism or pathogen (same species) that is to be “replaced” or “excluded”.

[00252] A relative stability in the microbial ecosystem of adults in the absence of gross perturbation has been suggested, and that long-term stability of human communities is not maintained by inertia, but by the action of restoring forces within a dynamic system. Relman, D.A., 2012, Nutr Rev., 70(Suppl 1): S2–S9.

[00253] Functional resilience is an intrinsic property of microbial communities and it has been suggested that state changes in response to environmental variation may be a key mechanism driving functional resilience in microbial communities. Song et al., 2015, *Frontiers in Microbiology*, 6, 1298. Seeking an integrated concept applicable to all microbial communities, Song et al. compared engineering and ecological resilience and reconciled them by arguing that resilience is an intrinsic property of complex adaptive systems which, after perturbation, recover their system-level functions and interactions with the environment, rather than their endogenous state.

[00254] Thus, a biome ecosystem has a dynamic but “stable elastoplastic equilibrium”. Once perturbed the biome “tries” to return to equilibrium. At any given moment the biome ecosystem has an equilibrium “base state”. Even under conditions of stress or massive cell death (e.g. washing, using ethanol, hand sanitizer, etc.) the biome is observed to typically regenerate in a similar form.

[00255] Microbiome ecosystems have “niches” defined by structure and internal and external interactions. One "fact" or "principal" of any biome structure is that different organisms occupy different "niches" in the biome, as defined/allowed by the structure of relationships. An ecological “niche” is the role and position a species has in its environment; how it meets its needs for food and shelter, how it survives, and how it reproduces. A species’ niche includes all of its interactions with the biotic and abiotic factors of its environment. A biome “niche” has specific environmental factors and conditions including, for example, pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, and electrolyte concentration.

[00256] Different organisms occupy different "niches" in the biome, as defined/allowed by the relationships structure. Niches as durable features of the biome ecosystem. Each niche has boundary conditions; a virtual shape or “footprint” reflecting the shape, which is discussed in the context of the “Hutchinsonian niche”.

[00257] The Hutchinsonian niche is an n-dimensional hypervolume, where the dimensions are environmental conditions and resources, that define the requirements of an individual or a species to practice "its" way of life, more particularly, for its population to persist. The "hypervolume" defines the multi-dimensional space of resources (e.g., light, nutrients, structure, etc.) available to (and specifically used by) organisms, and "all species other than those under consideration are regarded as part of the coordinate system."

[00258] A niche is a very specific segment of ecospace occupied by a single species. On the presumption that no two species are identical in all respects (i.e., Hardin's 'axiom of inequality') and the competitive exclusion principle, some resource or adaptive dimension will provide a niche specific to each species.

[00259] Niches are exclusive. Each organism competes with similar organisms for that niche, and the successful organism fills that niche. Two organisms do not/cannot fill the same niche because one will out-compete the other over time. Therefore, the coexistence of two organisms in the same biome over extended time periods means they do not fill the same niche.

[00260] Once a niche is left vacant, other organisms can fill that position. This is because one species does not have the same footprint as another species, so one species

cannot fill the same niche as another species. Successful replacement requires that the same organism (e.g., same species or close variant) should be used to fill or durably replace within a niche. It is recognized that partial competition exists in the form of transient colonization/infection and is an observable phenomenon.

[00261] Partial competition for a single niche can occur. One organism can “narrow” the “niche width” of another by partial competition. This might be the case with *Staphylococcus epidermidis* vs. *Staphylococcus aureus*. *S. epidermidis* is a commensal bacterium that secretes a serine protease capable of disassembling preformed *Staphylococcus aureus* biofilms, when used in high enough concentrations. Sugimoto *et al.*, *J Bacteriol*, 195(8) 1645-1655. However, there is an important distinction between an organism as a carrier of a toxic phenotypic expression (being temporarily massively overloaded by application at a site), vs that organism as a durable inhabitant of a niche that narrows or outcompetes the pathogen.

[00262] Interspecies co-colonization is a different phenomenon than the ability to durably fill and block an ecological niche. For example, Shu *et al.*, 2013 demonstrate that fermentation of glycerol to form short chain fatty acids (SCFA) with *Cutibacterium acnes* (*C. acnes*), a skin commensal bacterium that can inhibit growth of USA300, the most prevalent community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA). Shu demonstrates that SCFAs produced by *C. acnes* under anaerobic conditions inhibits *Staphylococcus aureus* growth in high concentrations. Shu *et al.*, 2013 PLoS ONE 8(2): e55380. However, these bacteria and this fermentation capability of *C. acnes* are already present in the normal human skin biome without there being effective eradication or diminution of *Staphylococcus aureus* pathogenicity. There is not any reason to believe that a hyper-physiologic application of these substrates would accomplish the goal of reduction of *Staphylococcus aureus* colonization or incidence of disease.

[00263] Decolonization / Recolonization

[00264] A method is provided to prevent or decrease recurrence of a pathogenic infection of a undesirable microorganism in a subject, comprising the steps of (i) suppressing (decolonizing) the undesirable microorganism on at least one site in the subject to reduce or eliminate the presence of the undesirable microorganism from the site; and (ii) replacing the undesirable microorganism by administering to the subject at

the at least one site a synthetic second microorganism having the same genus and species as the undesirable microorganism. Optionally, the method further comprises (iii) promoting colonization of the synthetic microorganism, for example, at the site of administration.

[00265] In some embodiments, the undesirable microorganism is a pathogenic microorganism and the term suppress (S) refers to a process of suppressing, reducing or eliminating the pathogenic microorganism at one or more, two or more, three or more, four or more sites in a subject. For example, the undesirable microorganism may be subject to nasal, mucosal, and/or dermal decolonization protocols.

[00266] The term replace (R) refers to replacing the pathogenic microorganism with a synthetic microorganism that is benign, drug-susceptible, and/or incapable of causing systemic or pathogenic infection in the subject. The replacement microorganism may be a molecularly modified synthetic microorganism of the same species as the pathogenic microorganism. The synthetic microorganism may be a molecularly modified microorganism of the same species, different strain, as the pathogenic microorganism, such that the synthetic microorganism is able to colonize the site on the subject, but is unable to cause systemic infection in the subject. By filling the vacated niche of the pathogenic microorganism, the synthetic microorganism is able to eliminate re-colonization by the pathogenic microorganism in the subject and thereby decrease or eliminate recurrence of pathogenic infection.

[00267] The term promote (P) refers to methods and compositions to promote replacement synthetic microorganism in the subject, for example, by employing prebiotics and biome management, for example, by employing a biome modulator in order to promote and support the new biome comprising the synthetic microorganism.

[00268] These methods broadly define a platform technology (SRP), with specifically designed protocols developed to address specific medical conditions (e.g. MRSA). If the processes of S, R, and P are selected properly – opening and then filling and sustaining a specific biome niche – a “durable” persistent biome is created that is capable of repelling pathogenic colonization.

[00269] A method is provided to decrease recurrence or chance of systemic infection of a pathogenic microorganism in a subject, the method comprising suppressing the pathogenic microorganism on the subject to significantly reduce or eliminate the

detectable presence of the pathogenic microorganism; and replacing the pathogenic microorganism by administering a synthetic microorganism to the subject, wherein the synthetic microorganism is capable of occupying the same niche as the pathogenic microorganism as evidenced by (1) having the same genetic background, or genus and species, as the pathogenic microorganism, and/or by (2) exhibiting durable detectable presence on the subject for at least 60 days following replacement. The method may include promoting the colonization of the synthetic microorganism on at least one site in the subject. In some cases, the subject may have been found to be colonized by the pathogenic microorganism.

[00270] Frequently, systemic infection of a subject with a pathogenic microorganism is preceded by colonization of the pathogenic microorganism in the subject. For example, a substantial proportion of cases of *Staphylococcus aureus* bacteremia appear to be of endogenous origin since they may originate from colonies in the nasal mucosa. For example, in one multicenter study of *Staphylococcus aureus* bacteremia, the blood isolates were identical to those from the anterior nares in 180 of 219 patients (82.2%). In a second study, 14 of 1278 patients who had nasal colonization with *Staphylococcus aureus* subsequently had *Staphylococcus aureus* bacteremia. In 12 of these 14 patients (86%), the isolates obtained from the nares were clonally identical to the isolates obtained from blood 1 day to 14 months later. See von Eiff et al., 2001, NEJM, vol. 344, No. 1, 11-16. Another study showed the relative risk of *Staphylococcus aureus* bacteremia was increased multi-fold in nasal carriers when compared to non-carriers, reporting an 80% match between the invasive isolate and previously found colonizing strain. Wertheim et al., Lancet 2004; 364: 703–705.

[00271] In some embodiments, the subject is found to be colonized with the pathogenic strain of the microorganism prior to systemic infection. In other embodiments, the subject may have been colonized or infected by a nosocomial (hospital-acquired) strain or community-acquired strain of a pathogenic microorganism.

[00272] The pathogenic microorganism may be a wild-type microorganism, and/or a pathogenic microorganism that may be colonized or detectably present in at least one site in the subject. The site may be a dermal or mucosal site in the subject. The one or sites of colonization may include skin and soft tissue including, but are not limited to, nares, throat, perineum, inguinal region, vagina, nasal, groin, perirectal area, finger webs, forehead, pharynx, axillae, hands, chest, abdomen, head, and/or toe webs.

[00273] The pathogenic microorganism may be a drug resistant microorganism. The Centers for Disease Control (CDC) recently published a report outlining the top 18 drug-resistant threats to the United States, see www.cdc.gov/drugresistance/biggest_threats. In some embodiments, the undesirable microorganism is selected from *Neisseria gonorrhoeae*, fluconazole-resistant *Candida*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus*, drug-resistant *Streptococcus pneumoniae* and drug-resistant tuberculosis, erythromycin-resistant Group A *Streptococcus*, and clindamycin-resistant Group B *Streptococcus*.

[00274] In some embodiments, the pathogenic microorganism is a MRSA.

[00275] The synthetic microorganism (a) must be able to fill the ecological niche in the at least one site in the subject so as to durably exclude the undesirable microorganism following suppression; and (b) must have at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter that is activated (induced) by a change in state in the environment compared to the normal physiological conditions in at least one site in the subject.

[00276] The synthetic microorganism may be of the same genus and species as the undesirable microorganism, in order to enhance the ability to fill the niche and durably exclude the undesirable microorganism in at least one site in the subject.

[00277] In some embodiments, the disclosure provides a synthetic microorganism that is not a pathogen and cannot become an accidental pathogen because it does not have the ability to infect the subject upon change in state, e.g., upon exposure to blood or serum. The synthetic microorganism comprises at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter that is activated (induced) by a change in state in the environment compared to the normal physiological conditions in at least one site in the subject. For example, if the site in the subject is a dermal or mucosal site, then exposure to blood or serum is a change in state resulting in cell death of the synthetic microorganism. For example, average cell death of the synthetic microorganism may occur within 6 hours, 5 hours, 4 hours, 2 hours, 90 minutes, 60 minutes, 45 minutes, 30 minutes, 20 minutes, 15 minutes, 10 minutes, 5 minutes, 2 minutes or 1 minute following change of state. The

change in state may be a change in one or more of the following conditions: pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, and/or electrolyte concentration from that in at least one site in a subject. In some embodiments, the change in state is a higher concentration of blood, serum, or plasma compared to normal physiological conditions at the at least one site in the subject.

[00278] In one embodiment, the pathogenic microorganism is a MRSA. MRSA is a variant subgroup of *Staphylococcus aureus*. MRSA strains typically include a *mecA* cassette that allows production of an alternate penicillin binding protein that render them resistant to treatment with most beta-lactam and other first-line antibiotics. *Staphylococcus aureus* as a whole (including MRSA) is present as part of the normal microbiome of approximately 30% of the total human population. As part of the microbiome *Staphylococcus aureus* lives most commonly on the surface of the skin and in the anterior nasal vestibules, but can also be found in smaller amounts in the deep oropharynx and gastrointestinal tract and as part of the normal vaginal flora in some individuals.

[00279] In the vast majority of individuals *Staphylococcus aureus* remains a non-invasive commensal bacterium merely occupying an ecologic niche and not causing disease. The colonization state is far more common than that of invasive disease – some researchers estimate this ratio to be on the order of 1000 to one. Laupland et al., J Infect Dis (2008) 198:336. However, in a fraction of those colonized this bacterium can cause disease either opportunistically or as a result of increased tendencies toward invasion due to the acquisition of genetic cassettes coding for virulence protein products that allow such strains to more effectively invade through the epidermal or mucosal tissue layers initiating deep infection. In both above circumstances, the presence of the *mecA* cassette limits the treatment options for these patients and a number of studies have documented the increased mortality rate associated with MRSA when compared to MSSA in bacteremia, endovascular infection and pneumonia.

[00280] Definitions

[00281] The singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[00282] The term "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items.

[00283] The terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. Unless otherwise defined, all terms, including technical and scientific terms used in the description, have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the event of conflicting terminology, the present specification is controlling.

[00284] The term "pathogen" or "pathogenic microorganism" refers to a microorganism that is capable of causing disease. A pathogenic microorganism may colonize a site on a subject and may subsequently cause systemic infection in a subject. The pathogenic microorganism may have evolved the genetic ability to breach cellular and anatomic barriers that ordinarily restrict other microorganisms. Pathogens may inherently cause damage to cells to forcefully gain access to a new, unique niche that provides them with less competition from other microorganisms, as well as with a ready new source of nutrients. Falkow, Stanley, 1998 *Emerging Infectious Diseases*, Vol. 4, No. 3, 495-497. The pathogenic microorganism may be a drug-resistant microorganism.

[00285] The term "virulent" or "virulence" is used to describe the power of a microorganism to cause disease.

[00286] The term "commensal" refers to a form of symbioses in which one organism derives food or other benefits from another organism without affecting it. Commensal bacteria are usually part of the normal flora.

[00287] The term "suppress" or "decolonize" means to substantially reduce or eliminate the original undesired pathogenic microorganism by various means (frequently referred to as "decolonization"). Substantially reduce refers to reduction of the undesirable microorganism by greater than 90%, 95%, 98%, 99%, or greater than 99.9% of original colonization by any means known in the art.

[00288] The term "replace" refers to replacing the original pathogenic microorganism by introducing a new microorganism (frequently referred to as "recolonization") that "crowds out" and occupies the niche(s) that the original

microorganism would ordinarily occupy, and thus preventing the original undesired microorganism from returning to the microbiome ecosystem (frequently referred to as “interference” and “non-co-colonization”).

[00289] The term “durably replace”, “durably exclude”, “durable exclusion”, or “durable replacement”, refers to detectable presence of the new synthetic microorganism for a period of at least 30 days, 60 days, 84 days, 120 days, 168 days, or 180 days after introduction of the new microorganism to a subject, for example, as detected by swabbing the subject. In some embodiments, “durably replace”, “durably exclude”, “durable exclusion”, or “durable replacement” refers to absence of the original pathogenic microorganism for a period of at least 30 days, 60 days, 84 days, 120 days, 168 days, or 180 days after introduction of the new synthetic microorganism to the subject, for example, absence as detected over at least two consecutive plural sample periods, for example, by swabbing the subject.

[00290] The term “promote”, or “promoting”, refers to activities or methods to enhance the colonization and survival of the new organism, for example, in the subject. For example, promoting colonization of a synthetic bacteria in a subject may include administering a nutrient, prebiotic, and/or probiotic bacterial species.

[00291] The terms "prevention", "prevent", "preventing", “prophylaxis” and as used herein refer to a course of action (such as administering a compound or pharmaceutical composition of the present disclosure) initiated prior to the onset of a clinical manifestation of a disease state or condition so as to prevent or reduce such clinical manifestation of the disease state or condition. Such preventing and suppressing need not be absolute to be useful.

[00292] The terms "treatment", "treat" and "treating" as used herein refers a course of action (such as administering a compound or pharmaceutical composition) initiated after the onset of a clinical manifestation of a disease state or condition so as to eliminate or reduce such clinical manifestation of the disease state or condition. Such treating need not be absolute to be useful.

[00293] The term "in need of treatment" as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient is ill, or will be ill, as the result of a condition that is treatable by a method, compound or pharmaceutical composition of the disclosure.

[00294] The term "in need of prevention" as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from prevention. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient will be ill or may become ill, as the result of a condition that is preventable by a method, compound or pharmaceutical composition of the disclosure.

[00295] The term "individual", "subject" or "patient" as used herein refers to any animal, including birds or mammals, such as mice, Norway rats, cotton rats, gerbils, caviae, hamsters, other rodents, rabbits, dogs, cats, swine, cattle, sheep, goat, horses, or primates, and humans. The term may specify male or female or both, or exclude male or female. In one aspect, the patient is an adult human. In another aspect, the patient is a non-neonate human infant. In another aspect, the patient is a human toddler, child, or adolescent. In some embodiments, the subject is found to be colonized with a pathogenic strain of a microorganism prior to a systemic infection, or the subject may have been colonized or infected by a nosocomial (hospital-acquired) strain or community-acquired strain of a pathogenic microorganism.

[00296] The term "neonate", or newborn, refers to an infant in the first 28 days after birth. The term "non-neonate" refers to an animal older than 28 days.

[00297] The term "effective amount" as used herein refers to an amount of an agent, either alone or as a part of a pharmaceutical composition, that is capable of having any detectable, positive effect on any symptom, aspect, or characteristics of a disease state or condition. Such effect need not be absolute to be beneficial.

[00298] The term "measurable average cell death" refers to the inverse of survival percentage for a microorganism determined at a predefined period of time after introducing a change in state compared to the same microorganism in the absence of a change in state under defined conditions. The survival percentage may be determined by any known method for quantifying live microbial cells. For example, survival percentage may be calculated by counting cfus/mL for cultured synthetic microorganism cells and counting cfus/mL of uninduced synthetic microorganism cells at the predefined period of time, then dividing cfus induced/mL by cfus/mL uninduced $\times 100 = x\%$ survival percentage. The measurable average cell death may be determined by $100\% - x\%$ survival percentage = $y\%$ measurable average cell death. For example, wherein the survival percentage is 5%, the measurable average cell death is $100\% - 5\% = 95\%$. Any method for counting cultured live microbial cells may be employed for calculation of

survival percentage including cfu, OD600, flow cytometry, or other known techniques. Likewise, an induced synthetic strain may be compared to a wild-type target microorganism exposed to the same conditions for the same period of time, using similar calculations to determine a “survival rate” wherein $100\% - \text{survival rate} = z\%$ “reduction in viable cells”.

[00299] The term "including" as used herein is non-limiting in scope, such that additional elements are contemplated as being possible in addition to those listed; this term may be read in any instance as "including, but not limited to."

[00300] The term "animal" refers to the animal kingdom definition.

[00301] The term "substantial identity" or "substantially identical," when referring to a nucleotide or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleotide (or its complementary strand), there is nucleotide sequence identity in at least about 95%, and more preferably at least about 96%, 97%, 98% or 99% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or Gap, as discussed below. A nucleotide molecule having substantial identity to a reference nucleotide molecule may, in certain instances, encode a polypeptide having the same or substantially similar amino acid sequence as the polypeptide encoded by the reference nucleotide molecule.

[00302] The term “derived from” when made in reference to a nucleotide or amino acid sequence refers to a modified sequence having at least 50% of the contiguous reference nucleotide or amino acid sequence respectively, wherein the modified sequence causes the synthetic microorganism to exhibit a similar desirable attribute as the reference sequence of a genetic element such as promoter, cell death gene, antitoxin gene, virulence block, or nanofactory, including upregulation or downregulation in response to a change in state, or the ability to express a toxin, antitoxin, or nanofactory product, or a substantially similar sequence, the ability to transcribe an antisense RNA antitoxin, or the ability to prevent or diminish horizontal gene transfer of genetic material from the undesirable microorganism. The term “derived from” in reference to a nucleotide sequence also includes a modified sequence that has been codon optimized for a particular microorganism to express a substantially similar amino acid sequence to that encoded by the reference nucleotide sequence. The term “derived from” when made in reference to a microorganism, refers to a target microorganism that is subjected to a molecular modification to obtain a synthetic

microorganism.

[00303] The term "substantial similarity" or "substantially similar" as applied to polypeptides means that two peptide or protein sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[00304] The term "conservative amino acid substitution" refers to wherein one amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties, such as charge or hydrophobicity. In general, a conservative amino acid substitution will not substantially change the functional properties of the, e.g., toxin or antitoxin protein. Examples of groups of amino acids that have side chains with similar chemical properties include (1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; (2) aliphatic-hydroxyl side chains: serine and threonine; (3) amide-containing side chains: asparagine and glutamine; (4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; (5) basic side chains: lysine, arginine, and histidine; (6) acidic side chains: aspartate and glutamate, and (7) sulfur-containing side chains are cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

[00305] Polypeptide sequences may be compared using FASTA using default or recommended parameters, a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (*see, e.g.,* Pearson, W.R., *Methods Mol Biol* 132: 185-219 (2000), herein incorporated by reference). Another preferred algorithm when comparing a sequence of the disclosure to a database containing a large number of sequences from different organisms is the computer program BLAST, especially BLASTP or TBLASTN, using default parameters. *See, e.g.,* Altschul *et al.*, *J Mol Biol* 215:403-410 (1990) and Altschul *et al.*, *Nucleic Acids Res* 25:3389-402 (1997).

[00306] Unless otherwise indicated, nucleotide sequences provided herein are presented in the 5' - 3' direction.

[00307] All pronouns are intended to be given their broadest meaning. Unless stated otherwise, female pronouns encompass the male, male pronouns encompass the

female, singular pronouns encompass the plural, and plural pronouns encompass the singular.

[00308] The term “systemic administration” refers to a route of administration into the circulatory system so that the entire body is affected. Systemic administration can take place through enteral administration (absorption through the gastrointestinal tract, e.g. oral administration) or parenteral administration (e.g., injection, infusion, or implantation).

[00309] The term “topical administration” refers to application to a localized area of the body or to the surface of a body part regardless of the location of the effect. Typical sites for topical administration include sites on the skin or mucous membranes. In some embodiments, topical route of administration includes enteral administration of medications or compositions.

[00310] The term “undesirable microorganism” refers to a microorganism which may be a pathogenic microorganism, drug-resistant microorganism, antibiotic-resistant microorganism, irritation-causing microorganism, odor-causing microorganism and/or may be a microorganism comprising an undesirable virulence factor.

[00311] The term “synthetic microorganism” refers to an isolated microorganism modified by any means to comprise at least one element imparting a non-native attribute. For example, the synthetic microorganism may be engineered to include a molecular modification comprising an addition, deletion and/or modification of genetic material to incorporate a non-native attribute. In some embodiments, the synthetic microorganism is not an auxotroph.

[00312] The term “detectable presence” of a microorganism refers to a confirmed positive detection in a sample of a microorganism genus, species and/or strain by any method known in the art. Confirmation may be a positive test interpretation by a skilled practitioner and/or by repeating the method.

[00313] The term “microbiome” or “microbiomic” or “microbiota” as used herein refers to microbiological ecosystems. These ecosystems are a community of commensal, symbiotic and pathogenic microorganisms found in and on all animals and plants.

[00314] The term “microorganism” as used herein refers to an organism that can be seen only with the aid of a microscope and that typically consists of only a single cell. Microorganisms include bacteria, protozoans and fungi.

[00315] The term “niche” and “niche conditions” as used herein refers to the ecologic array of environmental and nutritional requirements that are required for a particular species of microorganism. The definitions of the values for the niche of a species defines the places in the particular biomes that can be physically occupied by that species and defines the possible microbial competitors.

[00316] The term “colonization” as used herein refers to the persistent detectable presence of a microorganism on a body surface, e.g., a dermal or mucosal surface, without causing disease in the individual.

[00317] The term “co-colonization” as used herein refers to simultaneous colonization of a niche in a site on a subject by two or more strains, or variants within the same species of microorganisms. For example, the term “co-colonization” may refer to two or more strains or variants simultaneously and non-transiently occupying the same niche. The term non-transiently refers to positive identification of a strain or variant at a site in a subject over time at two or more time subsequent points in a multiplicity of samples obtained from the subject at least two weeks apart.

[00318] The term “bacterial replacement” or “non-co-colonization” as used herein refers to the principle that only one variant/strain of one species can occupy any given niche within the biome at any given time.

[00319] The term “kill switch” or “KS” as used herein refers to an intentional molecular modification of a synthetic microorganism, the molecular modification comprising a cell death gene operably linked to a regulatory region comprising an inducible promoter, genetic element or cassette, wherein induced expression of the cell death gene in the kill switch causes cell death, arrest of growth, or inability to replicate, of the microorganism in response to a specific state change such as a change in environmental condition of the microorganism.

For example, in the synthetic microorganism comprising a kill switch, the inducible first promoter may be activated by the presence of blood, serum, plasma, and/or heme, wherein the upregulation and transcription/expression of the operably associated cell death gene results in cell death of the microorganism or arrested growth of the microorganism so as to improve the safety of the synthetic microorganism.

[00320] The term “cell death gene” refers to a gene that when induced causes a cell to enter a state where it either ceases reproduction, alters regulatory mechanisms of the cell sufficiently to permanently disrupt cell viability, induces senescence, or induces

fatal changes in the genetic or proteomic systems of the cell. For example, the cell death gene may be a toxin gene encoding a toxin protein or toxin peptide.

[00321] The term “antitoxin gene” refers to a gene encoding an antitoxin RNA antisense molecule or an antitoxin protein or another antitoxin molecule specific for a cell death gene or a product encoded thereby

[00322] The term “virulence block” or “V-block” refers to a molecular modification of a microorganism that results in the organism have decreased ability to accept foreign DNA from other strains or species effectively resulting in the organism having decreased ability to acquire exogenous virulence or antibiotic resistance genes.

[00323] The term “nanofactory” as used herein refers to the molecular modification of a microorganism that results in the production of a product - either primary protein, polypeptide, amino acid or nucleic acid or secondary products of these modifications to beneficial effect.

[00324] The term “toxin protein” or “toxin peptide” as used herein refers to a substance produced internally within a synthetic microorganism in an effective amount to cause deleterious effects to the microorganism without causing deleterious effects to the subject that it colonizes.

[00325] The term “molecular modification” or “molecularly engineered” as used herein refers to an intentional modification of the genes of a microorganism using any gene editing method known in the art, including but not limited to recombinant DNA techniques as described herein below, NgAgo, mini-Cas9, CRISPR-Cpf1, CRISPR-C2c2, Target-AID, Lambda Red, Integrases, Recombinases, or use of phage techniques known in the art. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more elements, e.g., regulatory regions, promoters, toxin genes, antitoxin genes, or other domains into a suitable configuration, or to introduce codons, delete codons, optimize codons, create cysteine residues, modify, add or delete amino acids, etc. Molecular modification may include, for example, use of plasmids, gene insertion, gene knock-out to excise or remove an undesirable gene, frameshift by adding or subtracting base pairs to break the coding frame, exogenous silencing, e.g., by using inducible promoter or constitutive promoter which may be embedded in DNA encoding, e.g. RNA antisense antitoxin, production of CRISPR-cas9 or other editing proteins to digest, e.g., incoming virulence genes using guide RNA, e.g., linked to an inducible promoter or a constitutive promoter, or a

restriction modification/methylation system, e.g., to recognize and destroy incoming virulence genes to increase resistance to horizontal gene transfer. The molecular modification (e.g. kill switch, expression clamp, and/or v-block) may be durably incorporated to the synthetic microorganism by inserting the modification into the genome of the synthetic microorganism.

[00326] The synthetic microorganism may further comprise additional molecular modifications, (e.g., a nanofactory), which may be incorporated directly into the bacterial genome, or into plasmids, in order to tailor the duration of the effect of, e.g., the nanofactory production, and could range from short term (with non-replicating plasmids for the bacterial species,) to medium term (with replicating plasmids without addiction dependency) to long term (with direct bacterial genomic manipulation).

[00327] The molecular modifications may confer a non-native attribute desired to be durably incorporated into the host microbiome, may provide enhanced safety or functionality to organisms in the microbiome or to the host microbiome overall, may provide enhanced safety characteristics, including kill switch(s) or other control functions. In some embodiments the safety attributes so embedded may be responsive to changes in state or condition of the microorganism or the host microbiome overall.

[00328] The molecular modification may be incorporated to the synthetic microorganism in one or more, two or more, five or more, 10 or more, 30 or more, or 100 or more copies, or no more than one, no more than three, no more than five, no more than 10, no more than 30, or in no more than 100 copies.

[00329] The term “recurrence” as used herein refers to re-colonization of the same niche by a decolonized microorganism.

[00330] The term “pharmaceutically acceptable” refers to compounds, carriers, excipients, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00331] The term “pharmaceutically acceptable carrier” refers to a carrier that is physiologically acceptable to the treated subject while retaining the integrity and desired properties of the synthetic microorganism with which it is administered. Exemplary pharmaceutically acceptable carriers include physiological saline or phosphate-buffered saline. Other physiologically acceptable carriers and their

formulations are provided herein or are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (20th edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, Pa.

[00332] Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 5 to 6, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

[00333] All patents, patent publications, and peer-reviewed publications (i.e., "references") cited herein are expressly incorporated by reference to the same extent as if each individual reference were specifically and individually indicated as being incorporated by reference. In case of conflict between the present disclosure and the incorporated references, the present disclosure controls.

[00334] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

[00335] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

[00336] Vectors and Target Microorganisms

[00337] Also described herein are vectors comprising polynucleotide molecules, as well as target cells transformed with such vectors. Polynucleotide molecules described herein may be joined to a vector, which include a selectable marker and origin of replication, for the propagation host of interest. Target cells are genetically engineered to include these vectors and thereby transcribe RNA and express polypeptides. Vectors herein include polynucleotides molecules operably linked to suitable transcriptional or translational regulatory sequences, such as those for microbial target cells. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate

sequences which control transcription and translation. Nucleotide sequences as described herein are operably linked when the regulatory sequences herein functionally relate to, e.g., a cell death gene encoding polynucleotide.

[00338] Typical vehicles include plasmids, shuttle vectors, baculovirus, inactivated adenovirus, and the like. In certain examples described herein, the vehicle may be a modified pIMAY, pIMAYz, or pKOR integrative plasmid, as discussed herein.

[00339] A target microorganism may be selected from any microorganism having the ability to durably replace a specific undesirable microorganism after decolonization. The target microorganism may be a wild-type microorganism that is subsequently engineered to enhance safety by methods described herein. The target microorganism may be selected from a bacterial, fungal, or protozoal target microorganism. The target microorganism may be a strain capable of colonizing a dermal and/or mucosal niche in a subject. The target microorganism may be a wild-type microorganism, or a synthetic microorganism that may be subjected to further molecular modification. The target microorganism may be selected from a genus selected from the group consisting of *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Pseudomonas*. The target microorganism may be selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Escherichia coli*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*.

[00340] The target microorganism may be of the same genus and species as the undesirable microorganism, but of a different strain. For example, the undesirable microorganism may be an antibiotic-resistant *Staphylococcus aureus* strain, such as an MRSA strain. The antibiotic-resistant *Staphylococcus aureus* strain may be a pathogenic strain, which may be known to be involved in dermal infection, mucosal

infection, bacteremia, and/or endocarditis. Where the undesirable microorganism is a *Staphylococcus aureus* strain, e.g., an MRSA, the target microorganism may be, e.g., a less pathogenic strain which may be an isolated strain such as *Staphylococcus aureus* target cell such as an RN4220 or 502a strain, and the like. Alternatively, the target cell may be of the same strain as the undesirable microorganism. In another example, the undesirable microorganism is an *Escherichia coli* strain, for example, a uropathogenic *E. coli* type 1 strain or p-fimbriated strain, for example, a strain involved in urinary tract infection, bacteremia, and/or endocarditis. In another example, the undesirable strain is a *Cutibacterium acnes* strain, for example a strain involved in acnes vulgaris, bacteremia, and/or endocarditis. In another example, the undesirable microorganism is a *Streptococcus mutans* strain, for example, a strain involved in *S. mutans* endocarditis, dental caries.

[00341] Model Antibiotic-Susceptible Target Microorganism

[00342] The target microorganism may be an antibiotic-susceptible microorganism of the same species as the undesirable microorganism. In one embodiment, the undesirable microorganism is an MRSA strain and the replacement target microorganism is an antibiotic susceptible *Staphylococcus aureus* strain. The antibiotic susceptible microorganism may be *Staphylococcus aureus* strain 502a (“502a”). 502a is a coagulase positive, penicillin sensitive, nonpenicillinase producing staphylococcus, usually lysed by phages 7, 47, 53, 54, and 77. Serologic type (b)ci. Unusual disc antibiotic sensitivity pattern is exhibited by 502a because this strain is susceptible to low concentrations of most antibiotics except tetracycline; resistant to 5 µg, but sensitive to 10 µg of tetracycline. In some embodiments, the 502a strain may be purchased commercially as *Staphylococcus aureus* subsp. *Aureus* Rosenbach ATCC®27217™.

[00343] Unfortunately, even an antimicrobial agent-susceptible target microorganism may cause systemic infection. Therefore, as provided herein, the target microorganism is subjected to molecular modification to incorporate regulatory sequences including, e.g., an inducible first promoter for expression of the cell death gene, v-block, or nanofactory, in order to enhance safety and reduce the likelihood of pathogenic infection as described herein.

[00344] The target microorganism and/or the synthetic microorganism comprises (i) the ability to durably colonize a niche in a subject following decolonization of the undesirable microorganism and administering the target or synthetic microorganism to a subject, and (ii) the ability to prevent recurrence of the undesirable microorganism in the subject for a period of at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 24 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

[00345] Selection of a Target Microorganism for MRSA

[00346] Selection of the target microorganism may be performed by decolonizing the target microorganism and replacing with a putative target microorganism, as described herein. For example, the undesirable microorganism Methicillin-Resistant *Staphylococcus aureus* (MRSA) is the cause of a disproportionate amount of invasive bacterial infections worldwide. The colonization state for *Staphylococcus aureus* is regarded as a required precondition for most invasive infections. However, decolonization with standard antiseptic regimens has been studied as a method for reducing MRSA colonization and infections with mixed results. In one example provided herein, the feasibility and durability of a novel decolonization approach to undesirable microorganism MRSA by using intentional recolonization with a different *Staphylococcus aureus* strain as a candidate target microorganism was performed in hopes of improving duration of effect versus standard decolonization. Example 1 discloses the study in which a total of 765 healthy volunteers were screened for *Staphylococcus aureus* colonization. The overall MRSA rate for the screened population was 8.5%. A cohort of 53 MRSA colonized individuals participated in a controlled study of a decolonization/ recolonization therapy using *Staphylococcus aureus* 502a WT strain BioPlx-01 vs. a control group of standard decolonization alone. Duration of MRSA absence from the colonization state as well as persistence of the intentional MSSA recolonization was monitored for 6 months. The control group (n=15) for the efficacy portion of the MRSA decolonization protocol showed MRSA recurrence of 60% at the 4 week time point. The test group employing the BioPlx-01WT protocol (n=34) showed 0% MRSA recurrence at the 8 week primary endpoint and continued to show no evidence of MRSA recurrence out to 26 weeks. Instead these participants exhibited surprising persistence of colonization with MSSA likely

indicating ongoing colonization with the *Staphylococcus aureus* 502a BioPlx-01WT strain product out to 26 weeks. In addition, the components of the BioPlx-01WT in a phosphate buffered saline composition used in the decolonization/recolonization therapy showed no evidence of dermal irritation in a separate cohort of 55 participants. Therefore, target strain *Staphylococcus aureus* 502a BioPlx-01WT decolonization/recolonization protocol provides longer durability of decolonization from MRSA strains than standard decolonization and shows no observed negative dermal effects.

[00347] Methods for determining detectable presence of a microorganism

[00348] Any method known in the art may be employed for determination of the detectable presence of a microorganism genus, species and strain. An overview of methods may be found in Aguilera-Arreola MG. Identification and Typing Methods for the Study of Bacterial Infections: a Brief Review and Mycobacterial as Case of Study. Arch Clin Microbiol. 2015, 7:1, which is incorporated herein by reference.

[00349] The detectable presence of a genus, species and/or strain of a bacteria may be determined by phenotypic methods and/or genotypic methods. Phenotypic methods may include biochemical reactions, serological reactions, susceptibility to anti-microbial agents, susceptibility to phages, susceptibility to bacteriocins, and/or profile of cell proteins. One example of a biochemical reaction is the detection of extracellular enzymes. For example, staphylococci produce many different extracellular enzymes including DNAase, proteinase and lipases. Gould, Simon et al., 2009, The evaluation of novel chromogenic substrates for detection of lipolytic activity in clinical isolates of *Staphylococcus aureus* and MRSA from two European study groups. FEMS Microbiol Let 297; 10-16. Chromogenic substrates may be employed for detection of extracellular enzymes. For example, CHROMager™ MRSA chromogenic media (CHROMagar, Paris, France) may be employed for isolation and differentiation of Methicillin Resistant *Staphylococcus aureus* (MRSA) including low level MRSA. Samples are obtained from, e.g., nasal, perineal, throat, rectal specimens are obtained with a possible enrichment step. If the agar plate has been refrigerated, it is allowed to warm to room temperature before inoculation. The sample is streaked onto plate followed by incubation in aerobic conditions at 37 °C for 18-24 hours. The appearance of the colonies is read, wherein MRSA colonies appear as rose to mauve colored, Methicillin Susceptible *Staphylococcus aureus* (MSSA) colonies are inhibited, and other bacteria appear as blue, colorless or inhibited colonies. Definite identification as

MRSA requires, in addition, a final identification as *Staphylococcus aureus*. For example, CHROMagar™ Staph aureus chromogenic media may be employed where *S. aureus* appears as mauve, *S. saprophyticus* appears turquoise blue, *E. coli*, *C. albicans* and *E. faecalis* are inhibited. For detection of Group B *Streptococcus* (GBS) (*S. agalactiae*), CHROMagar™ StrepB plates may be employed, wherein *Streptococcus agalactiae* (group B) appear mauve, *Enterococcus* spp. and *E. faecalis* appear steel blue, *Lactobacilli*, *leuconostoc* and *lactococci* appear light pink, and other microorganisms are blue, colorless or inhibits. For detection of various *Candida* spp., CHROMager™ *Candida* chromogenic media may be employed. *Candida* species are involved in superficial oropharyngeal and urogenital infections. Although *C. albicans* remains a major species involved, other types such as *C. tropicalis*, *C. krusei*, or *C. glabrata* have increased as new antifungal agents have worked effectively against *C. albicans*. Sampling and direct streaking of skin, sputum, urine, vaginal specimens samples and direct streaking or spreading onto plate, followed by incubation in aerobic conditions at 30-37 °C for 48 hours, and reading of plates for colony appearance where *C. albicans* is green, *C. tropicalis* is metallic blue, *C. krusei* is pink and fuzzy, *C. kefyr* and *C. glabrata* are mauve-brown, and other species are white to mauve.

[00350] Genotypic methods for genus and species identification may include hybridization, plasmids profile, analysis of plasmid polymorphism, restriction enzymes digest, reaction and separation by Pulsed-Field Gel Electrophoresis (PFGE), ribotyping, polymerase chain reaction (PCR) and its variants, Ligase Chain Reaction (LCR), Transcription-based Amplification System (TAS), or any of the methods described herein.

[00351] Identification of a microbe can be performed, for example, by employing Galileo™ Antimicrobial Resistance (AMR) detection software (Arc Bio LLC, Menlo Park, CA and Cambridge, MA) that provides annotations for gram-negative bacterial DNA sequences.

[00352] The microbial typing method may be selected from genotypic methods including Multilocus Sequence Typing (MLST) which relies on PCR amplification of several *housekeeping* genes to create allele profiles; PCR-Extragenic Palindromic Repetitive Elements (rep-PCR) which involves PCR amplification of repeated sequences in the genome and comparison of banding patterns; AP-PCR which is Polymerase Chain Reaction using Arbitrary Primers; Amplified Fragment Length Polymorphism (AFLP) which involves enzyme restriction digestion of genomic DNA,

binding of restriction fragments and selective amplification; Polymorphism of DNA Restriction Fragments (RFLP) which involves Genomic DNA digestion or of an amplicon with restriction enzymes producing short restriction fragments; Random Amplified Polymorphic DNA (RAPD) which employs marker DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence; Multilocus Tandem Repeat Sequence Analysis (MLVA) which involves PCR amplification of loci VTR, visualizing the polymorphism to create an allele profile; or Pulsed-Fields Gel Electrophoresis (PFGE) which involves comparison of macro-restriction fragments. PFGE method of electrophoresis is capable of separating fragments of a length higher than 50 kb up to 10 Mb, which is not possible with conventional electrophoresis, which can separate only fragments of 100 bp to 50 kb. This capacity of PFGE is due to its multidirectional feature, changing continuously the direction of the electrical field, thus, permitting the re-orientation of the direction of the DNA molecules, so that these can migrate through the agarose gel, in addition to this event, the applied electrical pulses are of different duration, fostering the reorientation of the molecules and the separation of the fragments of different size. One PFGE apparatus may be the Contour Clamped Homogeneous Electric Fields (CHEF, BioRad). Pulsed-field gel electrophoresis (PFGE) is considered a gold standard technique for MRSA typing, because of its high discriminatory power, but its procedure is complicated and time consuming. The *spa* gene encodes a cell wall component of *Staphylococcus aureus* protein A, and exhibits polymorphism. The sequence based-*spa* typing can be used as a rapid test screen. Narukawa et al 2009 Tohoku J Exp Med 2009, 218, 207-213.

[00353] Methods and compositions are provided herein for suppressing (decolonizing) and replacing an undesirable microorganism with a new synthetic microorganism in order to durably displace and replace the undesirable microorganism from the microbiological ecosystem with a new microorganism so as to prevent the recurrence of the original undesirable organism (referred to here as niche or ecological interference).

[00354] In some embodiments, methods are provided to prevent colonization, prevent infection, decrease recurrence of colonization, or decrease recurrence of a pathogenic infection of a undesirable microorganism in a subject, comprising decolonization and administering a synthetic strain comprising a molecular modification

that decreases the ability of the synthetic microorganism to cause disease to the subject relative to the wild type target strain where the microorganism is selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*.

[00355] In some embodiments, a method is provided to prevent transmission by a subject, or recurrence of colonization or infection, of a pathogenic microorganism in a subject, comprising suppressing the pathogenic microorganism in the subject, and replacing the pathogenic microorganism by topically administering to the subject a composition comprising a benign microorganism of the same species, different strain. The method may further comprise promoting the colonization of the benign microorganism. In some embodiments, the benign microorganism is a synthetic microorganism having at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter, wherein the first promoter is activated in the presence of human serum or blood. In some embodiments, the first promoter is not activated during colonization of dermal or mucous membranes in a human subject.

[00356] In some embodiments, method is provided to prevent transmission by a subject, or recurrence of colonization or infection, of a methicillin-resistant *Staphylococcus aureus* (MRSA) in a subject, comprising suppressing the MRSA in the subject, and replacing the MRSA by topically administering to the subject a methicillin susceptible *Staphylococcus aureus* (MSSA) of the same species, different strain. The method may further comprise promoting the colonization of the MSSA in the subject.

[00357] A method is provided to prevent transmission by a subject, or recurrence of colonization or infection, of an undesirable microorganism in a subject, comprising suppressing the undesirable microorganism in the subject, and replacing the undesirable

microorganism by administering to the subject a second microorganism of the same species, different strain. The method may further comprise promoting the colonization of the second microorganism. In some embodiments, the undesirable microorganism is a drug-resistant pathogenic microorganism. In some embodiments, the second microorganism is a drug-susceptible microorganism. In some embodiments, the second microorganism is a synthetic microorganism.

[00358] Suppression/Decolonization

[00359] An undesirable microorganism may be suppressed, or decolonized, by topically applying a disinfectant, antiseptic, or biocidal composition directly to the skin or mucosa of the subject, for example, by spraying, dipping, or coating the affected area, optionally the affected area and adjacent areas, or greater than 25%, 50%, 75%, or greater than 90% of the external or mucosal surface area of the subject with the disinfectant, antiseptic, or biocidal composition. In some embodiments, the affected area, or additional surface areas are allowed to air dry or are dried with an air dryer under gentle heat, or are exposed to ultraviolet radiation or sunlight prior to clothing or dressing the subject. In one embodiment, the suppression comprises exposing the affected area, and optionally one or more adjacent or distal areas of the subject, with ultraviolet radiation. In various embodiments, any commonly employed disinfectant, antiseptic, or biocidal composition may be employed. In one embodiment, a disinfectant comprising chlorhexidine or a pharmaceutically acceptable salt thereof is employed.

[00360] In some embodiments, the bacteriocide, antiseptic, astringent, and/or antibacterial agent is selected from the group consisting of alcohols (ethyl alcohol, isopropyl alcohol), aldehydes (glutaraldehyde, formaldehyde, formaldehyde-releasing agents (noxythiolin = oxymethylenethiourea, tauroline, hexamine, dantoin), o-phthalaldehyde), anilides (triclocarban = TCC = 3,4,4'-trichlorocarbanilide), biguanides (chlorhexidine, alexidine, polymeric biguanides (polyhexamethylene biguanides with MW > 3,000 g/mol, vantocil), diamidines (propamidine, propamidine isethionate, propamidine dihydrochloride, dibromopropamidine, dibromopropamidine isethionate), phenols (fenchlor, *p*-chloro-*m*-xylenol, chloroxylenol, hexachlorophene), bis-phenols (triclosan, hexachlorophene), quaternary ammonium compounds (cetrimide, benzalkonium chloride, cetyl pyridinium chloride), silver compounds (silver sulfadiazine, silver nitrate), peroxy compounds (hydrogen peroxide, peracetic acid), iodine compounds (povidone-iodine, poloxamer-iodine, iodine), chlorine-releasing

agents (sodium hypochlorite, hypochlorous acid, chlorine dioxide, sodium dichloroisocyanurate, chloramine-T), copper compounds (copper oxide), botanical extracts (*Malaleuca* spp. (tea tree oil), *Cassia fistula* Linn, *Baekea frutesdens* L., *Melia azedarach* L., *Muntingia calabura*, *Vitis vinifera* L., *Terminalia avicennoides* Guill & Perr., *Phyllanthus discoideus* Muel. Muel-Arg., *Ocimum gratissimum* Linn., *Acalypha wilkesiana* Muell-Arg., *Hypericum pruinatum* Boiss.&Bal., *Hypericum olimpicum* L. and *Hypericum sabrum* L., *Hamamelis virginiana* (witch hazel), *Eucalyptus* spp., *rosemarinus officinalis* spp.(rosemary), *Thymus* spp.(thyme), *Lippia* spp. (oregano), *Cymbopogon* spp. (lemongrass), *Cinnamomum* spp., *Geranium* spp., *Lavendula* spp.), and topical antibiotic compounds (bacteriocins; mupirocin, bacitracin, neomycin, polymyxin B, gentamicin).

[00361] Suppression of the undesirable microorganism also may be performed by using photosensitizers instead of or in addition to, e.g., topical antibiotics. For example, Peng Zhang et al., Using Photosensitizers Instead of Antibiotics to Kill MRSA, GEN News Highlights, August 20, 2018; 48373, developed a technique using light to activate oxygen, which suppresses to microbial growth. Photosensitizers, such as dye molecules, become excited when illuminated with light. The photosensitizers convert oxygen into reactive oxygen species that kill the microbes, such as MRSA. In order to concentrate the photosensitizers to improve efficacy, water-dispersible, hybrid photosensitizers were developed by Zhang et al., comprising noble metal nanoparticles decorated with amphiphilic polymers to entrap molecular photosensitizers. The hybrid photosensitizers may be applied to a subject, for example, on a dermal surface or wound, in the form of a spray, lotion or cream, then illuminated with red or blue light to reduce microbial growth.

[00362] A decolonizing composition may be in the form of a topical solution, lotion, or ointment form comprising a disinfectant, biocide photosensitizer or antiseptic compound and one or more pharmaceutically acceptable carriers or excipients. In one specific example, an aerosol disinfectant spray is employed comprising chlorhexidine gluconate (0.4%), glycerin (10%), in a pharmaceutically acceptable carrier, optionally containing a dye to mark coverage of the spray. In one embodiment, the suppressing step comprises administration to one or more affected areas, and optionally one or more surrounding areas, with a spray disinfectant as disclosed in U.S. Pat. Nos. 4,548,807 and/or 4,716,032, each of which is incorporated herein by reference in its entirety. The

disinfectant spray may be commercially available, for example, Fight Bac®, Deep Valley Farm, Inc., Brooklyn, CT. Other disinfectant materials may include chlorhexidine or salts thereof, such as chlorhexidine gluconate, chlorhexidine acetate, and other diguanides, ethanol, SD alcohol, isopropyl alcohol, *p*-chloro-*o*-benzylphenol, *o*-phenylphenol, quaternary ammonium compounds, such as *n*-alkyl/dimethyl ethyl benzyl ammonium chloride/*n*-alkyl dimethyl benzyl ammonium chloride, benzalkonium chloride, cetrimide, methylbenzethonium chloride, benzethonium chloride, cetalkonium chloride, cetylpyridinium chloride, dofanium chloride, domiphen bromide, peroxides and permanganates such as hydrogen peroxide solution, potassium permanganate solution, benzoyl peroxide, antibacterial dyes such as proflavine hemisulphate, triphenylmethane, Brilliant green, Crystal violet, Gentian violet, quinolone derivatives such as hydroxyquinoline sulphate, potassium hydroxyquinoline sulphate, chlorquinaldol, dequalinium chloride, di-iodohydroxyquinoline, Burow's solution (aqueous solution of aluminum acetate), bleach solution, iodine solution, bromide solution. Various Generally Recognized As Safe (GRAS) materials may be employed in the disinfectant or biocidal composition including glycerin, and glycerides, for example but not limited to mono- and diglycerides of edible fat-forming fatty acids, diacetyl tartaric acid esters of mono- and diglycerides, triacetin, acetooleins, acetostearins, glyceryl lactopalmitate, glyceryl lactooleate, and oxystearins.

[00363] The suppression step –or decolonization- may be performed comprising administering 1-3 times daily, over a period of from 1 to 10 days; for example, on one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen or fourteen days. In other embodiments, the suppression step may be administered from two, three, four, five, or six times, each administration from 6 to 48 hours, 8 to 40 hours, 18 to 36 hours, or about 20 to 28 hours apart. In specific embodiments, the suppression step is administered once per day from one to five, or three to four consecutive days. In some embodiments, the suppression step does not include systemic administration of antimicrobial agents. In some embodiments, the suppression step does not include systemic administration of antibiotic, antiviral, or antifungal agents. In other embodiments, the suppression step includes systemic administration of antimicrobial agents. In some embodiments, the suppression step may include systemic administration of one or more antibiotic, antiviral, or antifungal agents.

[00364] Replace

[00365] Methods are provided wherein an undesirable microorganism is durably replaced with a synthetic microorganism. The synthetic microorganism has the ability to fill the same ecological niche and/or may be of the same species, different strain, as the pathogenic microorganism. By using same species, different strain, (or even the same strain) the environmental niche of the pathogenic microorganism may be filled, or durably replaced, with the benign synthetic microorganism.

[00366] Synthetic Microorganism

[00367] In some embodiments, the undesirable pathogenic microorganism is replaced with a synthetic microorganism. For example, the replacement strain may be a synthetic microorganism that is a molecularly modified strain of the same species as the undesirable or pathogenic microorganism or the same strain as the undesirable or pathogenic microorganism.

[00368] In some embodiments, a synthetic microorganism comprising a “kill switch” is provided exhibiting rapid and complete cell death on exposure to blood or serum, but exhibits normal metabolism and colonization function in other environments. In some embodiments, the synthetic microorganism comprises stable and immobile kill switch genes. The minimal kill switch (KS) components include a regulatory region (RR) containing operator, promoter and translation signals, that is strongly activated in response to blood or serum exposure, a kill switch gene expressing a toxic protein or RNA, and a means of transcription termination. Chromosomal integration of the KS is preferred. The chromosomal locus may be in a transcriptionally inactive region, for example, an intergenic region (IR) between a seryl-tRNA synthetase and an amino acid transporter. Insertions here do not affect transcription of flanking genes (Lei et al., 2012). Preferably, no known sRNAs are present in the IR. Any other inert loci may be selected.

[00369] The synthetic microorganism comprising a kill switch

[00370] In a particular embodiment, the pathogenic microorganism is an antimicrobial-resistant microorganism, and the replacement microorganism is a synthetic microorganism of the same species as the pathogenic microorganism. The synthetic microorganism may be a molecularly-modified, antibiotic-susceptible microorganism.

[00371] The synthetic microorganism may comprise one or more, two or more, or three or more molecular modifications comprising a first cell death gene operably linked to a first regulatory region comprising an inducible first promoter. Optionally, the

synthetic microorganism further comprises a second cell death gene operably linked to the first regulatory region comprising the first promoter or a second regulatory region comprising an inducible second promoter. The first promoter, and optionally the second promoter, is activated (induced) by a change in state in the microorganism environment compared to the normal physiological conditions at the at least one site in the subject. For example, the change in state may be selected from one or more changes in pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, and electrolyte concentration. In some embodiments, the change in state is a higher concentration of blood, serum, or plasma compared to normal physiological conditions at the at least one site in the subject.

[00372] In one specific embodiment, the pathogenic microorganism is a MRSA and the replacement microorganism is a synthetic microorganism that is a molecularly modified *Staphylococcus aureus* coagulase positive strain. The synthetic microorganism may be a molecularly modified *Staphylococcus aureus* 502a, as described herein.

[00373] The use of live *Staphylococcus aureus* as a therapeutic platform raises safety concerns because this pathogen can cause serious disease if it gains access to the circulatory system. In one embodiment, the synthetic microorganism is molecularly engineered to comprise a "kill switch" (KS) and an inducible promoter that induces rapid bacterial death upon exposure to whole blood or serum. The kill switch may be composed of DNA encoding 3 main components: i) "control region", containing a promoter and other regulatory sequences, that is strongly activated by blood or serum; ii) a toxic RNA or polypeptide, whose expression is driven by the control region, and; iii) a transcription terminator. A cassette composed of these elements maybe integrated into the *Staphylococcus aureus* chromosome at a site(s) amenable to alteration without adversely affecting bacterial function.

[00374] It is desirable that basal or "leaky" expression of the control region is minimized or avoided. For example, if significant mRNA production occurs before exposure to blood or serum, the strain could be weakened during manufacturing or skin colonization and may accumulate mutations that bypass or escape the KS. To address this, candidates are screened to find those that are strongly induced in serum, but also have very low or undetectable mRNA expression in standard growth media in vitro. Despite this effort, some leaky expression may be observed, which may be controlled by further comprising a iv) "expression clamp" to prevent untimely toxin production.

[00375] Recombinant Approach to Synthetic Microorganism

[00376] A synthetic microorganism is provided which comprises a recombinant nucleotide comprising at least one molecular modification (e.g., a kill switch) comprising (i) a cell death gene operatively associated with (ii) a first regulatory region comprising a first inducible promoter which is induced by a change in state in the environment of the synthetic microorganism. The synthetic microorganism may further comprises at least a second molecular modification (expression clamp) comprising (iii) an antitoxin gene specific for the first cell death gene, wherein the antitoxin gene is operably associated with (iv) a second regulatory region comprising a second promoter which is active (e.g., constitutive) upon dermal or mucosal colonization or in a media, and preferably is downregulated by change in state of the environment of the synthetic microorganism.

[00377] In some embodiments, a synthetic microorganism is provided comprising at least one molecular modification (e.g., a kill switch) comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter, wherein the first promoter is activated (induced) by a change in state in the microorganism environment compared to the normal physiological conditions at the at least one site in the subject, optionally wherein cell death of the synthetic microorganism occurs within 30, 60, 90, 120, 180, 360 or 240 minutes following change of state. The change in state may be selected from one or more conditions of pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, heme concentration, sweat concentration, sebum concentration, metal concentration, chelated metal concentration, change in composition or concentration of one or more immune factors, mineral concentration, and electrolyte concentration. In some embodiments, the change in state is a higher concentration of blood, serum, or plasma compared to normal physiological conditions at the at least one site in the subject.

[00378] Inducible promoters

[00379] A synthetic microorganism is provided which may comprise a recombinant nucleotide comprising at least one molecular modification (e.g., a kill switch) comprising (i) a cell death gene operatively associated with (ii) a first regulatory region comprising a first inducible promoter which exhibits conditionally high level gene expression of the recombinant nucleotide in response to exposure to blood, serum, or

plasma, of at least two fold, at least three fold, at least 10-fold, at least 20 fold, at least 50 fold, at least 100-fold increase of basal productivity.

[00380] The inducible first promoter may be activated (induced) upon exposure to an increased concentration of blood, serum, plasma, or heme after a period of time, e.g., after 15 minutes, 30 minutes, 45 minutes, 90 minutes, 120 minutes, 180 minutes, 240 minutes, 360 minutes, or any time point in between, to increase transcription and/or expression at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, at least 100-fold, at least 300-fold, or at least 600-fold compared to transcription and/or expression in the absence of blood, serum, plasma or heme (non-induced).

[00381] The blood or serum inducible first promoter may be selected by a process comprising selecting a target microorganism, selecting one or more first promoter candidate genes in the target microorganism, growing the microorganism in a media, obtaining samples of the microorganism at $t=0$ min, adding serum or blood to the media, obtaining samples at $t=n$ minutes, where $n=1-240$ min or more, 15-180 min, or 30-120 min, performing RNA sequencing of the samples, and comparing RNA sequencing read numbers for candidate first promoter in samples obtained at $t=0$ min, and $t=n$ minutes after exposure to blood or serum for the candidate first promoter gene. Alternatively, samples obtained after $t=n$ minutes after exposure to blood or serum may be compared to $t=n$ minutes in media without blood or serum for the candidate first promoter. Candidate first promoters may be selected from those that exhibit upregulation by RNA sequencing after target cell growth at $t=n$ min in blood or serum of greater than about 10-fold, greater than about 20-fold, greater than about 50-fold, greater than about 100-fold, or greater than about 500-fold, when compared to the candidate promoter in the target cell at $t=0$, or when compared to the candidate promoter in the target cell at $t=n$ in media without serum or blood.

[00382] Several serum responsive promoter candidate genes in *Stapylocooccus aureus* 502a were upregulated by greater than 20-fold after exposure to serum for 30 minutes as determined by RNA sequencing as compared to $t=0$ including *isdB* gene CH52_00245 (479-fold), *sbnB* gene CH52_05135 (158-fold), *isdC* gene CH52_00235 (93-fold), *sbnA* gene CH52_05140 (88-fold), *srtB* gene CH52_00215 (73-fold), *sbnE* gene CH52_05120 (70-fold), *sbnD* gene CH52_05125 (66-fold), *isdI* gene CH52_00210 (65-fold), heme ABC transporter 2 gene CH52_00225 (65-fold), *sbnC* gene CH52_05130 (63-fold), heme ABC transporter gene CH52_00230 (60-fold), *isd* ORF3

gene CH52_00220 (51-fold), sbnF gene CH52_05115 (43 fold), alanine dehydrogenase gene CH52_11875 (43-fold), HarA gene CH52_10455 (43-fold), sbnG gene CH52_05110 (42-fold), diaminopimelate decarboxylase gene CH52_05105 (32-fold), iron ABC transporter gene CH52_05145 (31-fold), threonine dehydratase gene CH52_11880 (24-fold), and isdA gene CH52_00240 (21-fold).

[00383] Several serum responsive promoter candidate genes in target microorganism *Staphylococcus aureus* 502a were found to be upregulated by greater than 20-fold after exposure to serum for 30 minutes as determined by RNAseq compared to TSB at 30 minutes including isdB gene CH52_00245 (471-fold), isdC gene CH52_00235 (56-fold), isdI gene CH52_00210 (53-fold), sbnD gene CH52_05125 (52-fold), sbnC gene CH52_05130 (51-fold), sbnE gene CH52_05120 (50-fold), srtB gene CH52_00215 (47-fold), sbnB gene CH52_05135 (44-fold), sbnF gene CH52_05115 (44- fold), heme ABC transporter 2 gene CH52_00225 (43-fold), isdA gene CH52_00240 (40-fold), heme ABC transporter gene CH52_00230 (40-fold), sbnA gene CH52_05140 (37-fold), isd ORF3 gene CH52_00220 (35-fold), sbnG gene CH52_05110 (34-fold), HarA gene CH52_10455 (28-fold), diaminopimelate decarboxylase gene CH52_05105 (25-fold), sbnI gene CH52_05100 (22-fold), and alanine dehydrogenase gene CH52_11875 (20-fold). Iron ABC transporter gene CH52_05145 was upregulated (19-fold) after 30 min of exposure to serum compared to 30 min in TSB. Threonine dehydratase gene CH52_11880 was upregulated (14-fold) after 30 min of exposure to serum compared to 30 min in TSB.

[00384] Several serum responsive promoter candidate genes in target microorganism *Staphylococcus aureus* 502a were upregulated by greater than 50-fold after exposure to serum after 90 minutes as determined by RNAseq compared to t=0 including isdB gene CH52_00245 (2052-fold), sbnB gene CH52_05135 (310-fold), alanine dehydrogenase gene CH52_11875 (304-fold), sbnE gene CH52_05120 (190-fold), sbnD gene CH52_05125 (187-fold), isdC gene CH52_00235 (173-fold), sbnC gene CH52_05130 (162-fold), sbnA gene CH52_05140 (143-fold), srtB gene CH52_00215 (143-fold), sbnG gene CH52_05110 (133-fold), sbnF gene CH52_05115 (129- fold), heme ABC transporter gene CH52_00230 (125-fold), heme ABC transporter 2 gene CH52_00225 (117-fold), isdI gene CH52_00210 (115-fold), HarA gene CH52_10455 (114-fold), diaminopimelate decarboxylase gene CH52_05105 (102-fold), sbnI gene CH52_05100 (101-fold), isd ORF3 gene CH52_00220 (97-fold), SAM dep

Metrans gene CH52_04385 (75-fold). Iron ABC transporter gene CH52_05145 (44-fold), *isdA* gene CH52_00240 (44-fold), and siderophore ABC transporter gene CH52_05150 (33-fold) were also upregulated after 90 min exposure to serum compared to t=0.

[00385] Several serum responsive promoter candidate genes in target microorganism *Staphylococcus aureus* 502a were found to be upregulated by greater than 50-fold after exposure to serum after 90 minutes as determined by RNA sequencing compared to growth in TSB at 90 minutes including *isdB* gene CH52_00245 (1240-fold), *sbnD* gene CH52_05125 (224-fold), heme ABC transporter gene CH52_00230 (196-fold), *sbnE* gene CH52_05120 (171-fold), *srtB* gene CH52_00215 (170-fold), *isdC* gene CH52_00235 (149-fold), *sbnC* gene CH52_05130 (147-fold), diaminopimelate decarboxylase gene CH52_05105 (141-fold), heme ABC transporter 2 gene CH52_00225 (135-fold), *sbnB* gene CH52_05135 (130-fold), *sbnF* gene CH52_05115 (127-fold), *bnG* gene CH52_05110 (120-fold), *isd* ORF3 gene CH52_00220 (119-fold), *isdI* gene CH52_00210 (118-fold), *HarA* gene CH52_10455 (117-fold), *isdA* gene CH52_00240 (115-fold), *sbnA* gene CH52_05140 (93-fold), and *sbnI* gene CH52_05100 (89-fold). Iron ABC transporter gene CH52_05145 (47-fold), siderophore ABC transporter gene CH52_05150 (37-fold), and SAM dep Metrans gene CH52_04385 (25-fold) were also upregulated after 90 min exposure to serum compared to TSB at t=90 min.

[00386] The blood or serum inducible first promoter genes for use in a *Staphylococcus aureus* synthetic microorganism may be selected from or derived from a gene selected from *isdA* (iron-regulated surface determinant protein A), *isdB* (iron-regulated surface determinant protein B), *isdG* (heme-degrading monooxygenase), *hlgA* (gamma-hemolysin component A), *hlgA1* (gamma-hemolysin), *hlgA2* (gamma-hemolysin), *hlgB* (gamma-hemolysin component B), *hrtAB* (heme-regulated transporter), *sbnC* (luc C family siderophore biosynthesis protein), *sbnE* (lucA/lucC family siderophore biosynthesis protein), *lrgA* (murein hydrolase regulator A), *lrgB* (murein hydrolase regulator B), *ear* (Ear protein), *fhuA* (ferrichrome transport ATP-binding protein *fhuA*), *fhuB* (ferrichrome transport permease), *hly* (phospholipase C), *splF* (serine protease *SplF*), *splD* (serine protease *SplD*), *dps* (general stress protein 20U), SAUSA300_2617 (putative cobalt ABC transporter, ATP-binding protein), SAUSA300_2268 (sodium/bile acid symporter family protein), SAUSA300_2616

(cobalt family transport protein), srtB (Sortase B), sbnA (probable siderophore biosynthesis protein sbnA), leuA (2-isopropylmalate synthase amino acid biosynthetic enzyme), sstA (iron transport membrane protein), sirA (iron ABC transporter substrate-binding protein), IsdA (heme transporter), and Spa (Staphylococcal protein A), HlgA (gamma hemolysin), leuA(amino acid biosynthetic enzyme), sstA (iron transporter), sirA (iron transport), spa (protein A), or IsdA (heme transporter) , or a substantially identical gene. The first promoter genes also may be selected from the group consisting of SAUSA300_0119 (Ornithine cyclodeaminase family protein), lrgA (Murein hydrolase transporter), and bioA (Adenosylmethionine-8-amino-7-oxononanoate aminotransferase), or a substantially identical gene.

[00387] The blood or serum blood or serum inducible first promoter genes for use in a *Staphylococcus aureus* synthetic microorganism may be selected from or derived from a gene selected from isdB gene CH52_00245, sbnD gene CH52_05125, heme ABC transporter gene CH52_00230, sbnE gene CH52_05120, srtB gene CH52_00215, isdC gene CH52_00235, sbnC gene CH52_05130, diaminopimelate decarboxylase gene CH52_05105, heme ABC transporter 2 gene CH52_00225, sbnB gene CH52_05135, sbnF gene CH52_05115, bnG gene CH52_05110, isd ORF3 gene CH52_00220, isdI gene CH52_00210, HarA gene CH52_10455, isdA gene CH52_00240, sbnA gene CH52_05140, and sbnI gene CH52_05100, iron ABC transporter gene CH52_05145, siderophore ABC transporter gene CH52_05150, and SAM dep Metrans gene CH52_04385.

[00388] The blood or serum indicible first promoter gene for use in a *Staphylococcus aureus* synthetic microorganism may be derived from or comprise a nucleotide sequence selected from 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, and 163, or a substantially identical sequence.

[00389] In one embodiment, the synthetic microorganism is a molecularly modified *Staphylococcus aureus* 502a. Raw sequences of first ORF in the operon that follows each regulatory region, from start codon to stop codon, used for design of real time PCR probes are shown in Table 2.

[00390] Table 2. *Staphylococcus aureus* strain 502a, raw sequences of first ORF in the operon that follows each regulatory region used for design of real time PCR probes.

<i>Staphylococcus aureus</i> strain 502a, <i>spa</i> ORF of 502a	ATGACTTTACAAATACATACAGGGGGTATTAATTT GAAAAAGAAAAACATTTATTCAATTCGTAACTAGGTGTAGGTATTGCAT CTGTAACCTTTAGGTACATTACTTATATCTGGTGGCGTAACACCTGCTGCA AATGCTGCGCAACACGATGAAGCTCAACAAAATGCTTTTTATCAAGTGTT AAATATGCCTAACTTAAACGCTGATCAACGTAATGGTTTTATCCAAAGCC TTAAAGATGATCCAAGCCAAAGTGCTAACGTTTTAGGTGAAGCTCAAAAA CTTAATGACTCTCAAGCTCCAAAAGCTGATGCGCAACAAAATAAATTCAA CAAAGATCAACAAAGCGCCTTCTATGAAATCTTGAACATGCCTAACTTAA ACGAAGCGCAACGTAACGGCTTCATTCAAAGTCTTAAAGACGACCCAAGC CAAAGCACTAATGTTTTAGGTGAAGCTAAAAAATTAAACGAATCTCAAGC ACCGAAAGCTGATAACAATTTCAACAAAGAACAACAAAATGCTTTCTATG AAATCTTGAATATGCCTAACTTAAACGAAGAACAACGCAATGGTTTCATC CAAAGCTTAAAGATGACCCAAGCCAAAGTGCTAACCTATTGTCAGAAGC TAAAAAGTTAAATGAATCTCAAGCACCGAAAGCGGATAACAAATTCAACA AAGAACAACAAAATGCTTTCTATGAAATCTTACATTTACCTAACTTAAAC GAAGAACAACGCAATGGTTTCATCCAAAGCTTAAAGATGACCCAAGCCA AAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCACAAGCAC CAAAAGCTGACAACAAATTCAACAAAGAACAACAAAATGCTTTCTATGAA ATTTTACATTTACCTAACTTAACTGAAGAACAACGTAACGGCTTCATCCA AAGCCTTAAAGACGATCCTTCAGTGAGCAAAGAAATTTTAGCAGAAGCTA AAAAGCTAAACGATGCTCAAGCACCAAAAGAGGAAGACAACAAAAAACCT GGTAAAGAAGACGGCAACAAGCCTGGTAAAGAAGACAACAAAAAACCTGG TAAAGAAGACGGCAACAAGCCTGGTAAAGAAGACAACAACAAACCTGGCA AAGAAGACGGCAACAAGCCTGGTAAAGAAGACAACAACAAGCCTGGTAAA GAAGACGGCAACAAGCCTGGTAAAGAAGACGGCAACAAACCTGGTAAAGA AGACGGCAACGGAGTACATGTCGTTAAACCTGGTGATACAGTAAATGACA TTGCAAAAGCAAACGGCACTACTGCTGACAAAATTGCTGCAGATAACAAA TTAGCTGATAAAAAACATGATCAAACTGGTCAAGAACTTGTTGTTGATAA GAAGCAACCAGCAAACCATGCAGATGCTAACAAAGCTCAAGCATTACCAG AAACTGGTGAAGAAAATCCATTATCGGTACAACGTGATTTGGTGGATTA TCATTAGCCTTAGGTGCAGCGTTATTAGCTGGACGTCGTCGCAACTATA A SEQ ID NO: 1
<i>Staphylococcus aureus</i> strain 502a, <i>sirA</i> ORF of 502a	ATGAATAAAGTAATTAAAATGC TTGTTGTTACGCTTGCTTTTCTACTTGTTTTAGCAGGATGTAGTGGGAAT TCAAATAAACAATCATCTGATAACAAAGATAAGGAAACAACCTTCAATTAA ACATGCAATGGGTACAACGTAAATTAAGGGAAACCAAGCGTGTTGTTA CGCTATATCAAGGTGCCACTGACGTCGCTGTATCTTTAGGTGTTAAACCT GTAGGTGCTGTAGAATCATGGACACAAAACCGAAATTCGAATACATAAA AAATGATTTAAAAGATACTAAGATTGTAGGTCAAGAACCTGCACCTAACT TAGAGGAAATCTCTAAATTAAAACCGGACTTAATTGTCGCGTCAAAAGTT AGAAATGAAAAAGTTTACGATCAATTATCTAAAATCGCACCAACAGTTTC TACTGATACAGTTTTCAAATTCAAAGATACAACCTAAGTTAATGGGGAAAG CTTTAGGGAAAGAAAAAGAGCTGAAGATTTACTTAAAAAGTACGATGAT AAAGTAGCTGCATTCAAAAAGATGCAAAAGCAAAGTATAAAGATGCATG GCCATTGAAAGCTTCAGTTGTTAACTTCCGTGCTGATCATACAAGAATTT ATGCTGGTGGATATGCTGGTGAAATCTTAAATGATTTAGGATTCAAACGT AATAAAGACTTACAAAAACAAGTTGATAATGGTAAAGATATTATCCAACCT TACATCTAAAGAAAGCATTCCATTAAATGAACGCTGATCATATTTTTGTAG TAAAAATCAGATCCAAATGCGAAAGATGCTGCATTAGTTAAAAAGACTGAA AGCGAATGGACTTCAAGTAAAGAGTGGAAAAATTTAGACGCAGTTAAAAA CAACCAAGTATCTGATGATTTAGATGAAATCACTTGGAACCTAGCTGGCG GATATAAATCTTCATTAATAACTTATTGACGATTTATATGAAAAGTTAAAT ATTGAAAAACAATCAAAATAA

	SEQ ID NO: 2
<i>Staphylococcus aureus</i> strain 502a, <i>sstA</i> of 502a	ATGATAATGATTATCATTAATTTAA AGGGAGAAAAATTTGTAATGAAGTATTTATTAAAGGGAAATATTTTGCTT CTATTACTAATATTGTTGACAATTATTTTCGTTGTTTCATAGGTGTGAGTGA ACTATCAATTAAAGATTTACTACATTTAACTGAATCACAGCGGAATATTT TATTCTCAAGCCGAATACCAAGGACGATGAGTATTTTAAATTGCTGGAAGT TCGTTGGCTTTAGCAGGCTTGATAATGCAACAAATGATGCAAAATAAGTT TGTTAGTCCGACTACAGCTGGAACGATGGAATGGGCTAAACTAGGTATTT TAATTGCTTTATTGTTCTTTCCAACCGGTCATATTTTATTAAACTAGTA TTTGCTGTTATTTGCAGTATTTGCGGTACGTTTTTATTGTTAAATCAT TGATTTTATAAAAGTGAAAGATGTCATTTTGTACCGCTTTTAGGAATTA TGATGGGTGGGATTGTTGCAAGTTTCACAACCTTCATCTCATTGCGCACG AATGCTGTTCAAAGCATTGGTAACTGGCTTAACGGGAACTTTGCCATTAT CACAACTGGACGCTATGAAATTTTATATTTAAGTATTCCTCTTTTAGCAT TGACATATCTTTTGTCTAATCATTTACGATTGTAGGAATGGGTAAAGAC TTTACTAATAATTTAGGTTTGAGTTACGAAAAATTAATTAACATCGCATT GTTTATTACTGCAACTATTACAGCATTGGTAGTGGTGACTGTTGGAACAT TACCGTTCTTAGGACTAGTAATACCAAATATTATTTCAATTTATCGAGGT GATCATTTGAAAAATGCTATCCCTCATACGATGATGTTAGGTGCCATCTT TGTATTATTTTCTGATATAGTTGGCAGAATTGTTGTTTATCCATATGAAA TAAATATTGGTTTAACAATAGGTGTATTTGGAACAATCATTTTCCTTATC TTGCTTATGAAAGGTAGGAAAAATTATGCGCAACAATAA SEQ ID NO: 3
<i>Staphylococcus aureus</i> strain 502a, <i>hlgA</i> ORF of 502a	ATGAACT TAAAAATTAAATAGAAAGAAAGTGATTTCTATGATTAAAAATAAAATATTA ACAGCAACTTTAGCAGTTGGTTTAATAGCCCCTTTAGCCAATCCATTTAT AGAAATTTCTAAAGCAGAAAAATAAGATAGAAGATATCGGTCAAGGTGCAG AAATCATCAAAAGAACACAAGACATTACTAGCAAACGATTAGCTATAACT CAAAACATTCAATTTGATTTTGTAAGATAAAAAATATAACAAAGATGC CCTAGTTGTTAAGATGCAAGGCTTCATCAGCTCTAGAACAACATATTCAG ACTTAAAAAATATCCATATATTAAAAGAATGATATGGCCATTTCAATAT AATATCAGTTTGAAAACGAAAGACTCTAATGTTGATTTAATCAATTATCT TCCTAAAAATAAAATTGATTCAGCAGATGTTAGTCAGAAATTAGGCTATA ATATCGGCGGAACTTCCAATCAGCGCCATCAATCGGAGGCAGTGGCTCA TTCAACTACTCTAAACAATTAGTTATAATCAAAAAAATATGTTACTGA AGTAGAAAGTCAGAACTCTAAAGGTGTTAAATGGGGAGTGAAAGCAAATT CATTTGTTACACCGAATGGTCAAGTATCTGCATATGATCAATACTTATTT GCACAAGACCCAACTGGTCCAGCAGCAAGAGACTATTTTCGTCCCAGATAA TCAATTACCTCCTTTAATTCAAAGTGGCTTTAATCCATCATTTATTACAA CATTGTCACACGAAAGAGGTAAAGGTGATAAAGCGAGTTTGAAATCACT TACGGCAGAAACATGGATGCTACATATGCTTACGTGACAAGACATCGTTT AGCCGTTGATAGAAAACATGATGCTTTTAAAAACCGAAACGTTACAGTTA AATATGAAGTGAAGTGGAAAACACATGAAGTAAAAATTAAAAGCATCACA CCTAAGTAA SEQ ID NO: 4
<i>Staphylococcus aureus</i> strain	ATGACAAAACATTATTTAAACAGTAAGTATCAATC AGAACAACGTTTCATCAGCTATGAAAAAGATTACAATGGGTACAGCATCTA TCATTTTAGGTTCCCTTGATACATAGGCGCAGACAGCCAACAAGTCAAT GCGGCAACAGAAGCTACGAACGCAACTAATAATCAAAGCACACAAGTTTC

502a, <i>isdA</i> ORF of 502a	TCAAGCAACATCACAACCAATTAATTTCCAAGTGCAAAAAGATGGCTCTT CAGAGAAGTCACACATGGATGACTATATGCAACACCCTGGTAAAGTAATT AAACAAAATAATAAATATTATTTCCAAACCGTGTTAAACAATGCATCATT CTGGAAAAGATACAAATTTTACAATGCAAACAATCAAGAATTAGCAACAA CTGTTGTTAACGATAATAAAAAAGCGGATACTAGAACAATCAATGTTGCA GTTGAACCTGGATATAAGAGCTTAACACTAAAGTACATATTGTCGTGCC ACAAATTAATTACAATCATAGATATACTACGCATTTGGAATTTGAAAAAG CAATTCCTACATTAGCTGACGCAGCAAAACCAAACAATGTAAACCGGTT CAACCAAAACCAGCTCAACCTAAAACACCTACTGAGCAAACTAAACCAGT TCAACCTAAAGTTGAAAAAGTTAAACCTACTGTAACTACAACAAGCAAAG TTGAAGACAATCACTCTACTAAAGTTGTAAGTACTGACACAACAAAAGAT CAAACCTAAAACACAACTGCTCATAACAGTTAAAACAGCACAACTGCTCA AGAACAAAATAAAGTTCAAACACCTGTTAAAGATGTTGCAACAGCGAAAT CTGAAAGCAACAATCAAGCTGTAAGTGATAATAAATCACAACAACTAAC AAAGTTACAAAACATAACGAAACGCCTAAACAAGCATCTAAAGCTAAAGA ATTACCAAAAACCTGGTTTAACTTCAGTTGATAACTTTATTAGCACAGTTG CCTTCGCAACACTTGCCCTTTTAGGTTCAATTATCTTTATTACTTTTCAA AGAAAAGAATCTAAATAA SEQ ID NO: 5
<i>Staphylococcus aureus</i> strain 502a, <i>leuA</i> of 502a	ATGAGTAGTCATATTCAAATTTTTGATACGACACTAAGAGACGGTGAACA AACACCAGGAGTGAATTTTACTTTTGATGAACGCTTGCGTATTGCATTGC AATTAGAAAAATGGGGTGTAGATGTTATTGAAGCTGGATTTCTGTCTCA AGTACAGGTAGCTTTAAATCTGTTCAAGCAATTGCACAAACATTAACAAC AACGGCTGTATGTGGTTTAGCTAGATGTAAAAATCTGACATCGATGCTG TATATGAAGCAACAAAAGATGCAGCGAAGCCGGTCGTGCATGTTTTTATA GCAACATCACCTATTCATCTTGAACATAAACTTAAATGTCTCAAGAAGA CGTTTTAGCATCTATTAAAGAACATGTCACATACGCGAAACAATTATTTG ACGTTGTTCAATTTTACCTGAAGATGCAACGCGTACTGAATTACCATTCT TTAGTGAATGTGTACAACTGCCGTTGACGCTGGAGCTACAGTTATTAA TATTCCTGATACAGTCGGCTACAGTTACCATGATGAATATGCACATATTT TCAAAACCTTAACAGAATCTGTAAACATCTCAAATGAAATTATTTATAGT GCTCATTGCCATGACGATTTAGGAATGGCTGTTTCAAATAGTTTAGCTGC AATTGAAGCGGTGCGAGACGAATTGAAGGCACTGTAAATGGTATTGGTG AACGAGCAGGTAATGCAGCACTTGAAGAAGTCGCGCTTGCACTATACGTT CGAAATGATCATTATGGTGCTCAAACCTGCCCTTAATCTCGAAGAACTAA AAAAACATCGGATTTAATTTCAAGATATGCAGGTATTCGAGTGCCTAGAA ATAAAGCAATTGTTGGCCAAAATGCATTTAGTCATGAATCAGGTATTCAC CAAGATGGCGTATTAAACATCGTGAAACATATGAAATTATGACACCTCA ACTTGTGGTGTAAAGCAGACTGAACTTCCATTAGGAAAATTATCTGGTA AACACGCCTTCTCAGAGAAGTTAAAAGCATTAGGTTATAACATTGATAAA GAAGCGCAAATAGATTTATTTAAACAATTCAAGACCATTGCGGACAAAAA GAAATCTGTTTCAGATAGAGATATTCATGCGATTATTCAAGGTTCTGAGC ATGAGCATCAAGCACTTTATAAATTGGAAACACTACAACCTACAATATGTC TCTAGCGCCTTCAAAGTGCTGTTGTTGTTGTTAAAGATAAAGAGGGTCA TATTTACCAGGATTCAAGTATTGGTACTGGTTCAATCGTAGCAATTTACA ATGCAGTTGATCGTATTTTCCAGAAAGAAACAGAATTAATTGATTATCGT ATTAATTCTGTCACTGAAGGTACTGATGCCCAAGCAGAAGTACATGTA TTTATTGATTGAAGGTAAGACTGTCAATGGCTTTGGTATTGATCATGATA TTTTACAAGCCTCTTGTAAGCATACGTAGAAGCACATGCTAAATTTGCA GCTGAAAATGTTGAGAAGGTAGGTAAT SEQ ID NO: 6

[00391] As discussed herein below, the synthetic microorganism may include an expression clamp molecular modification that prevents expression of the cell death gene, wherein the expression clamp comprises an antitoxin gene specific for the cell death gene operably associated with a second promoter which is active upon dermal or mucosal colonization or in TSB media, and is preferably downregulated in blood, serum or plasma, for example, the second promoter may comprise a *clfB* gene (clumping factor B), for example as shown in Table 3.

[00392] Table 3. Other Sequences Used for Design of Real time PCR probes

<i>clfB</i> ORF of 502a (to drive antitoxin for "expression clamp")	ATGAAAAAAAAAGAATTGATTATTTGTCTGAATAAGCAGAATAAGTATTCGAT TAGACGTTTTACAGTAGGTACCACATCAGTAATAGTAGGGGCAACTATAC TATTTGGGATAGGCAATCATCAAGCACAAGCTTCAGAACAAATCGAACGAT ACAACGCAATCTTCGAAAAATAATGCAAGTGCAGATTCCGAAAAAACAA TATGATAGAAACACCTCAATTAATAACAACGGCTAATGATACATCTGATA TTAGTGCAAACACAAACAGTGC GAATGTAGATAGCACAAACAAACCAATG TCTACACAAACGAGCAATACCACTACAACAGAGCCAGCTTCAACAAATGA AACACCTCAACCGACGGCAATTAAAAATCAAGCAACTGCTGCAAAAATGC AAGATCAAACCTGTTCTCTCAAGAAGCAAATTCTCAAGTAGATAATAAACAA ACGAATGATGCTAATAGCATAGCAACAAACAGTGAGCTTAAAAATTCTCA AACATTAGATTTACCACAATCATCACCACAAACGATTTCCAATGCGCAAG GAACTAGTAAACCAAGTGTTAGAACGAGAGCTGTACGTAGTTTAGCTGTT GCTGAACCGGTAGTAAATGCTGCTGATGCTAAAGGTACAAATGTAAATGA TAAAGTTACGGCAAGTAATTTCAAGTTAGAAAAGACTACATTTGACCCTA ATCAAAGTGGTAACACATTTATGGCGGCAAATTTTACAGTGACAGATAAA GTGAAATCAGGGGATTATTTTACAGCGAAGTTACCAGATAGTTTAACTGG TAATGGAGACGTGGATTATTCTAATTCAAATAATACGATGCCAATTGCAG ACATTAAAAGTACGAATGGCGATGTTGTAGCTAAAGCAACATATGATATC TTGACTAAGACGTATACATTTGTCTTTACAGATTATGTAAATAATAAAGA AAATATTAACGGACAATTTTCAATTACCTTTATTTACAGACCGAGCAAAGG CACCTAAATCAGGAACATATGATGCGAATATTAATATTGCGGATGAAATG TTTAATAATAAAATTACTTATAACTATAGTTCGCCAATTGCAGGAATTGA TAAACCAAATGGCGCGAACATTTCTTCTCAAATTATTGGTGTAGATACAG CTTCAGGTCAAACACATACAAGCAAACAGTATTTGTTAACCCTAAGCAA CGAGTTTTAGGTAATACGTGGGTGTATATTAAAGGCTACCAAGATAAAAT CGAAGAAAGTAGCGGTAAAGTAAGTGCTACAGATACAAAACCTGAGAATTT TTGAAGTGAATGATACATCTAAATTATCAGATAGCTACTATGCAGATCCA AATGACTCTAACCTTAAAGAAGTAACAGACCAATTTAAAAATAGAATCTA TTATGAGCATCCAAATGTAGCTAGTATTAAATTTGGTGATATTACTAAAA CATATGTAGTATTAGTAGAAGGGCATTACGACAATACAGGTAAGAACCTTA AAAACCTCAGGTTATTCAAGAAAAATGTTGATCCTGTAACAAATAGAGACTA CAGTATTTTCGGTTGGAATAATGAGAATGTTGTACGTTATGGTGGTGGAA GTGCTGATGGTGATTACAGCAGTAAATCCGAAAGACCAACTCCAGGGCCG CCGGTTGACCCAGAACCAAGTCCAGACCCAGAACCAGAACCAACGCCAGA TCCAGAACCAAGTCCAGACCCAGAACCAGCAACCAAGCCAGACCCGGATC CGGATTCGGATTACAGACAGTGACTCAGGCTCAGACAGCGACTCAGGTTCA GATAGCGACTCAGAATCAGATAGCGATTCCGATTACAGACAGTGATTACAGA TTCAGACAGCGACTCAGAATCAGATAGCGATTACAGAATCAGATAGCGACT CAGATTCAGATAGCGATTACAGATTCAGATAGCGATTACAGAATCAGATAGC GATTCGGATTACAGACAGTGATTACAGATTCAGACAGCGACTCAGAATCAGA TAGCGACTCAGAATCAGATAGTGAGTCAGATTCAGACAGTGACTCGGACT CAGACAGTGATTACAGACTCAGATAGCGATTACAGACTCAGATAGCGATTCA GACTCAGACAGCGATTACAGATTACAGACAGCGACTCAGAATCAGACAGCGA
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	CTCAGACTCAGATAGCGACTCAGACTCAGACAGCGACTCAGATTCAGATA GCGATTGAGACTCAGACAGCGACTCAGACTCAGACAGCGACTCAGACTCA GATAGCGATTGAGACTCAGACAGCGACTCAGATTGAGATAGCGATTGCGA CTCAGACAGCGATTGAGATTGAGACAGCGACTCAGACTCGGATAGCGATT CAGATTGAGACAGCGACTCAGACTCGGATAGCGACTCGGATTGAGATAGT GACTCCGATTCAAGAGTTACACCACCAATAATGAACAGAAAAGCACCATC AAATCCTAAAGGTGAAGTAAACCATTCTAATAAGGTATCAAAACAACACA AAACTGATGCTTTACCAGAAAACAGGAGATAAGAGCGAAAACACAAATGCA ACTTTATTTGGTGCAATGATGGCATTATTAGGATCATTACTATTGTTTAG AAAACGCAAGCAAGATCATAAAGAAAAAGCGTAAATACTTTTTTAGGCCG AATACATTTGTATTCGGTTTTTTTTGTTGAAAATGATTTTAAAGTGAATTG SEQ ID NO: 7
<i>gyrA</i> ORF of 502a (internal housekeeping gene)	ATGGCTGAATTACCTCAATCAAGAATAAATGAACGAAATATTACCAGTGA AATGCGTGAATCATTTTTTAGATTATGCGATGAGTGTTATCGTTGCTCGTG CATTGCCAGATGTTTCGTGACGGTTTAAAACCAGTACATCGTCGTATACTA TATGGATTAAATGAACAAGGTATGACACCGGATAAATCATATAAAAAATC AGCACGTATCGTTGGTGACGTAATGGGTAAATATCACCTCATGGTGACT CATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTGAGTTATCGT TATCCGCTTGTTGATGGCCAAGGTAACCTTTGGTTCAATGGATGGAGATGG CGCAGCAGCAATGCGTTTATACTGAAGCGCGTATGACTAAAATCACACTTG AACTGTTACGTGATATTAATAAAGATACAATAGATTTTATCGATAACTAT GATGGTAATGAAAGAGAGCCGTCAGTCTTACCTGCTCGATTCCCTAACTT GTTAGCCAATGGAGCATCAGGTATAGCGGTAGGTATGGCAACGAATATTC CACCACATAACTTAACAGAATTAATCAATGGTGTACTTAGCTTAAGTAAG AACCCTGATATTTCAATTGCTGAGTTAATGGAGGATATTGAAGGTCCTGA TTTCCCAACTGCTGGACTTATTTTAGGTAAGAGTGGTATTAGACGTGCAT ATGAAACAGGTCGTGGTTCAATTCAAATGCGTTCTCGTGCAGTTATTGAA GAACGTGGAGGCGGACGTCAACGTATTGTTGTCACTGAAATTCCTTTCCA AGTGAATAAGGCTCGTATGATTGAAAAAATTGCAGAGCTCGTTTCGTGACA AGAAAAATTGACGGTATCACTGATTTACGTGATGAAACAAGTTTACGTACT GGTGTGCGTGTCGTTATTGATGTGCGTAAGGATGCAAATGCTAGTGTCAT TTTAAATAACTTATACAAACAAACACCTCTTCAAACATCATTGTTGTTGA ATATGATTGCACTTGTAATGGTAGACCGAAGCTTATTAATTTAAAAGAA GCGTTGGTACATTATTTAGAGCATCAAAGACAGTTGTTAGAAGACGTAC GCAATACAACCTTACGTAAAGCTAAAGATCGTGCCACATTTTAGAAGGAT TACGTATCGCACTTGACCATATCGATGAAATTATTTCAACGATTCGTGAG TCAGATACAGATAAAGTTGCAATGGAAAGCTTGCAACAACGCTTCAAAC TTCTGAAAAACAAGCTCAAGCTATTTTAGACATGCGTTTAAAGACGTCTAA CAGGTTTAGAGAGAGACAAAATTGAAGCTGAATATAATGAGTTATTAAT TATATTAGTGAATTAGAAACAATCTTAGCTGATGAAGAGTATTACTACA ATTAGTTAGAGATGAATTAACAGAAATTCGAGATCGTTTCGGTGATGATC GTCGTACTGAAATCCAATTAGGTGGATTGGAAGATTTAGAAGATGAAGAT CTCATTCCAGAAGAACAATTTGAATTACACTAAGCCATAATAACTACAT TAAACGTTTGCCGGTATCTACATATCGTGCTCAAAACCGTGGTGGTCGTG GTGTTCAAGGTATGAATACATTGGAAGAAGATTTTGTCACTCAATTGGTA ACTTTAAGTACACATGACCATGTATTGTTCTTTACTAACAAAGGTCGTGT ATACAACTTTAAAGGTTATGAAGTGCCTGAGTTATCAAGACAGTCTAAAG GTATTCCTGTAGTGAATGCTATTGAACTTGAAAATGATGAAGTCATTAGT ACAATGATTGCTGTTAAAGACCTTGAAAGTGAAGACAACCTCTTAGTGTT TGCAACTAAACGTGGTGTGCTTAAACGTTGAGCATTAAGTAACCTCTCAA GAATAAATAGAAATGGTAAGATTGCGATTTGTTTCAGAGAAGATGATGAG TTAATTGCAGTTCGCTTAACAAGTGGTCAAGAAGATATCTTGATTGGTAC ATCACATGCATCATTAATTCGATTCCCTGAATCAACATTACGTCCTTTAG GCCGTACAGCAACGGGTGTGAAAGGTATTACACTTCGTGAAGGTGACGAA GTTGTAGGGCTTGATGTAGCTCATGCAACAGTGTTGATGAAGTATTAGT AGTTACTGAAATGGTTATGGTAAACGTACGCCAGTTAATGACTATCGTT

	TATCAAATCGTGGTGGTAAAGGTATTAAAACAGCTACGATTACTGAGCGT AATGGTAATGTTGTATGTATCACTACAGTAACTGGTGAAGAAGATTTAAT GATTGTTACTAATGCAGGTGTCATTATTCGACTAGATGTTGCAGATATTT CTCAAAATGGTCGTGCAGCACAAAGGTGTCGCTTAATTGCTTAGGTGAT GATCAATTTGTTTCAACGGTTGCTAAAGTAAAAGAAGATGCAGAAGATGA AACGAATGAAGATGAGCAATCTACTTCAACTGTATCTGAAGATGGTACTG AACACAACGTGAAGCGGTTGTAAATGATGAAACACCAGGAAATGCAATT CATACTGAAGTGATTGATTGATTCAGAAGAAAATGATGAAGATGGACGTATTGA AGTAAGACAAGATTTTCATGGATCGTGTTGAAGAAGATATACAACAATCAT CAGATGAAGATGAAGAATAATAA SEQ ID NO: 8
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[00393] Additional oligonucleotides used in the recombinant approach to preparing the synthetic microorganism molecularly modified *Staphylococcus aureus* 502a are shown in Table 4A shown in FIG. 3A-C, and promoter sequences are shown below.

[00394] Cell Death Genes

[00395] The synthetic microorganism may contain a kill switch molecular modification comprising cell death gene operably associated with an inducible first promoter, as described herein. The cell death gene may be selected from any gene, that upon overexpression results in cell death or significant reduction in the growth of the synthetic microorganism within a predefined period of time, preferably within 15 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 240 minutes, or 360 minutes of induction.

[00396] Cell death genes, toxin genes, or kill switch genes, have been developed in other contexts.

[00397] WO 2016/210373, Jonathan Kotula et al., assigned to Synlogic, Inc. discloses a recombinant bacterial cell that is an auxotroph engineered for biosafety, for example, that comprises a repression based kill switch gene that comprises a toxin, an anti-toxin and an arabinose inducible promoter and depends on the presence of an inducer (e.g., arabinose) to keep cells alive.

[00398] US 8,975,061, Bielinski, discloses regulation of toxin and antitoxin genes for biological containment for preventing unintentional and/or uncontrolled spread of the microorganisms in the environment.

[00399] WO 1999/058652, Gerdes, discloses cytotoxin based biological containment and kill systems including *E. coli* relBE locus and similar systems found in Gram-negative and Gram-positive bacteria and Archae.

[00400] US 20150050253, Gabant, discloses controlled growth of microorganisms and controlling the growth/spread of other exogenous recombinant or other microbes.

[00401] WO 2017/023818 and WO 2016/210384, Falb, disclose bacteria engineered to treat disorders involving propionate metabolism.

[00402] US 20160333326, Falb, discloses bacteria engineered to treat diseases associated with hyperammonemia.

[00403] US 9101597, Garry, discloses immunoprotective primary mesenchymal stem cells and methods and a proapoptotic kill switch is described for use in mesenchymal stem cells.

[00404] US 20160206666, Falb, discloses bacteria engineered to treat diseases that benefit from reduced gut inflammation and/or tighten gut mucosal barrier.

[00405] In some embodiments, synthetic microorganisms are provided that comprise one or more of SprA1 (*Staphylococcus aureus*), Sma1 (*Serratia marcescens*), RelF (*E. coli*), KpnI (*K. pneumoniae*) and/or RsaE (*Staphylococcus aureus*) toxin genes.

[00406] In the present disclosure, various cell death toxin genes were tested in combinations with previously identified optimal control regions: i) a 30 amino acid peptide (PepA1) that forms pores in the cell membrane, impairing its function; ii) a restriction enzyme (KpnI or other) that rapidly digests the bacterial chromosome; iii) a small RNA (RsaE) that impairs central biochemical metabolism by inhibiting translation of 2 essential genes; iv) a restriction endonuclease (Sma1) derived from *Serratia marcescens*; and v) a toxin gene derived from *E. coli* (RelF). Some toxins are more potent than others and the ideal combination of control region induction strength and toxin potency may result in a strain that is healthy at baseline and that rapidly dies in the circulatory system.

[00407] *sprA1* (*Staphylococcus aureus*) toxin gene (encoding PepA1 peptide) is described in WO 2013/050590, Felden, B, and Sayed, N, disclosing use of PepA1 as an

antimicrobial, but the focus is on using the peptide as purified exogenous therapeutic to be delivered into the body.

[00408] *relF* (*E. coli*) toxin gene is described in EP 20090168998, Gerdes, disclosing kill switches for the purpose of biocontainment and focuses on revolve around killing of Gram-negative bacteria.

[00409] *relF* toxin gene is described in US 8852916, Hyde and Roderick, disclosing mechanisms of triggering cell death of microorganisms (programmed cell death). The main application is to use RelF in environmental biocontainment.

[00410] *relF* is described in US 8682619, Amodei, prophetically discloses RelF to regulate bacterial population growth.

[00411] The synthetic microorganism may be derived from a *Staphylococcus aureus* target microorganism by insertion of a kill switch molecular modification comprising a regulatory region comprising an inducible promoter operably linked to a cell death gene which may be a toxin gene.

[00412] The cell death gene may be selected from or derived from a *sprA1* gene (encoding a peptide toxin that forms pores in cell membrane), *sprA2* gene, *sprG* gene, *smal* gene (a restriction endonuclease), *kpnI* gene (restriction enzyme that rapidly digests bacterial chromosome), *rsaE* gene (a small RNA that impairs central metabolism by inhibiting translation of 2 essential genes), a *relF* gene (*E. coli*), *yoeB* gene, *mazF* gene, *yefM* gene, or lysostaphin toxin gene. The synthetic *Staphylococcus aureus* may include a kill switch molecular modification comprising a cell death gene having a nucleotide sequence selected from SEQ ID NOs: 122, 124, 125, 126, 127, 128, 274, 275, 284, 286, 288, 290, 315, or 317, or a substantially identical nucleotide sequence.

[00413] In a specific embodiment, a synthetic *Staphylococcus aureus* is provided having a molecular modification comprising a blood or serum inducible first promoter operably associated with a cell death gene comprising or derived from a *SprA1* gene.

[00414] Multiple kill switches

[00415] One KS may be sufficient to equip the synthetic microorganism with the desired characteristics, but more than one KS may further enhance the strain by: i) dramatically reducing the rate of KS-inactivating mutations, and; ii) killing the cell by more than one pathway, which could cause faster cell death (a product-enhancing

feature). The cell death gene may comprise one or more of the DNA sequences (7) downstream of promoters that are shown below. Base pair numbers correspond to pCN51 vector location.

[00416] 1. The *sprAI* gene sequence between restriction sites PstI and EcoRI is shown below. The sequence was synthesized by DNA 2.0(Atum) and ligated into a vector, which can be transformed into E.coli cells for replication. The *sprAI* gene was restriction cut at PstI and EcoRI sites and isolated by gel electrophoresis. Full sequence between restriction sites with possible start and stop sites italicized.

PstI

CTGCAGGG TACCGCAGAG AGGAGGTGTA

6101 TAAGGTG***ATG*** CTTATTTTCG TTCACATCAT AGCACCAGTC ATCAGTGGCT

6151 GTGCCATTGC GTTTTTTTCT TATTGGCTAA GTAGACGCAA TACAAAA***TAG***

6201 GTGACATATA GCCGCACCAA TAAAAATCCC CTCACTACCG CAAATAGTGA

6251 GGGGATTGGT GTATAAGTAA ATACTTATTT TCGTTGTGGA TCCTTGACTG

6301 AATTC SEQ ID NO: 122

EcoRI

[00417] 2. The DNA sequence for the regulatory RNA *sprAI**sprAI*_{AS} (*sprAI**sprAI* antisense) under the ClfB promoter (which is cloned in reverse behind the *sprAI* gene, including the antisense regulatory RNA). This DNA sequence produces a non-coding antisense regulatory RNA, which acts as an antitoxin by regulating the translation of *sprAI* outside of the environmental factors of serum and/or blood. Below is the *sprAI**sprAI*_{AS} DNA sequence.

EcoRI

GAATTCAGTCAAGGATCCACAACGAAAATAAGTATTTACTTATACACCAATCCCCTCACTATTTGCGGTA

GTGAGGGGATTTTTATTGGTGCGGCTATATGTCACCTATTTTGTATTGCGTCTACTTAGCCAATAAGAAA

AAAACGCAATGGCACAGCCACTGATGACTGGTGCTATGATGTGAACGAAAATAAGCATCACCTTATACAC

CTCCTCTCTGCGGTACCCTGCAG SEQ ID NO: 123

PstI

[00418] 3. The *SmaI* DNA sequence between restriction sites PstI and EcoRI. Sequence was synthesized by DNA 2.0(Atum) and ligated into a vector that can be transformed into *E. coli* cells for replication. *SmaI* gene was restriction cut at PstI and EcoRI sites and isolated by gel electrophoresis. Full sequence between restriction sites with start and stop sites italicized.

C TGCAG**ATG**AG

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5751 CAGGGATGAC CAACTCTTTA CACTTTGGGG AAAGCTTAAC GATCGTCAGA
5801 AGGATAATTT TCTAAAATGG ATGAAAGCTT TTGATGTAGA GAAAACTTAC
5851 CAAAAACAA GTGGGGATAT TTTCAATGAT GATTTTTTCG ATATATTTGG
5901 TGATAGATTA ATTACTCATC ATTTTCAGTAG CACGCAAGCT TTAACAAAAA
5951 CTTTATTCGA ACATGCTTTT AATGACTCCT TAAATGAATC TGGAGTTATA
6001 TCCTCTCTTG CGGAAAGTAG AACAAACCTT GGGCATGACA TAACAATCGA
6051 TAGCATAAAG GTTGCTTTAA AAACAGAAGC AGCTAAAAAT ATTAGCAAAT
6101 CATATATTCA TGTAAGTAAG TGGATGGAGT TAGGCAAGGG GGAGTGGATT
6151 CTAGAATTAT TATTAGAACG GTTTTTCAGAG CATCTAGAGA ATTATGAACG
6201 TATTTTCACA CTCAGATATT TTAAAATATC CGAGTATAAA TTTAGCTACC
6251 AGCTTGTAGA AATACCCAAG AGTCTTTTGT TGGAAAGCAA AAATGCGAAA
6301 TTAGAAATAA TGTCGGGAAG CAAACAAAGC CCTAAGCCCG GCTATGGATA
6351 TGTGTTAGAT GAAAATGAAA ATAAGAAGTT TTCTCTATAC TTTGATGGTG
6401 GTGCCGAGAG AAAACTTCAA ATAAACATT TAAATTTAGA ACATTGCATT
6451 GTTCATGGAG TTTGGGATTT TATTCTACCG CCGCCTTAAG AATTC SEQ ID NO: 124

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[00419] 4. The *rsaE* DNA sequence between restriction sites PstI and EcoRI. Sequence was synthesized by DNA 2.0(Atum) and ligated into a vector that can be transformed into *E. coli* cells for replication. RsaE small regulatory RNA (sRNA) was restriction cut at PstI and EcoRI sites and isolated by gel electrophoresis. This contains a 5' run-in and the mature RNA is processed out starting at the bold GAAATTAA and ending at the stretch of Ts after the ACG.

CTGCAGAT GGTAGAGATA GCATGTTATA

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6101 TTATGAACAT GAAATTAATC ACATAACAAA CATACCCCTT TGTTTGAAGT
6151 GAAAAATTTT TCCCATCCCC TTTGTTTAGC GTCGTGTATT CAGACACGAC
6201 GTTTTTTTGA ATTC SEQ ID NO: 125

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[00420] 5. A variant can be used for RsaE sRNA which may express the sRNA more highly which may work more effectively. This variant would start with the GAAATTAA at the 5' end.

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6110 GAAATTAATC ACATAACAAA CATACCCCTT TGTTTGAAGT
6151 GAAAAATTTT TCCCATCCCC TTTGTTTAGC GTCGTGTATT CAGACACGAC
6201 GTTTTTTTGA ATTC SEQ ID NO: 126

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[00421] 6. The *relF* (*E. coli*) DNA sequence. This potential kill gene will be tested and cloned.

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ATGAAGCAGC AAAAGGCGAT GTTAATCGCC CTGATCGTCA TCTGTTTAAC
CGTCATAGTG
ACGGCACTGG TAACGAGGAA AGACCTCTGC GAGGTACGAA TCCGAACCGG

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CCAGACGGAG

GTCGCTGTCT TCACAGCTTA CGAACCTGAG GAGTAA SEQ ID NO: 127

[00422] 7. The *KpnI* (restriction enzyme from *K. pneumoniae*) DNA sequence will be tested and cloned.

atggatgtctttgataaagtttatagtgatgataataatagttatgaccaaaaaactgtaagtcagcgtattgaagccct
atttcttaataaccttggcaagttgtaactcgtcagcaaatcattaggcgaggcaactgatccaaaaacagggaaacaac
cagaaaattggcatcagagactttcagaactacgaactgataaaggatatactattttatcctggcgggatgaaggtt
ttagctccgaagagtataatgccacacgcaacaagacgcccgaaggcagcaaaagcgtgtattaccgacaaaagaaac
ctgggaacaggttttgatagagctaattactcttgcgagtggcaggaagatggtaacactgtgggttagttgaaggtg
atattgatcctatagggggaggcacggtcaaactaacaccagaccatatgacacctcattcaatagatcccgcaactgat
gtaaatgatcctaaaatgtggcaagcattgtgtggacgtcatcaagttatgaaaaaaattattgggattcaaataatgg
gaaaataaatgtcattggtatattgcagtcagtaaatgagaaacaaaagaatgatgcttagagtttctttgaattatt
atggattgaaaagataa SEQ ID NO: 128

[00423] A synthetic *Staphylococcus aureus* 502a is provided herein comprising at least one molecular modification (kill switch) comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter, optionally wherein the first cell death gene comprises a nucleotide sequence selected from SEQ ID NO: 122, 124, 125, 126, 127, 128, 274, 275, 284, 286, 288, 290, 315, and 317, or a substantially identical nucleotide sequence

[00424] Although kill switches (KSs) have been described for other purposes, the present KS has the unique features: i) it responds to being exposed to blood or serum; ii) it is endogenously regulated, meaning that the addition or removal of small molecules is not needed to activate or tune the KS (not an auxotroph); and iii) useful combinations of control region/toxin, and of multiple such cassettes may be used to achieve superior performance.

[00425] Expression Clamp

[00426] A synthetic microorganism is provided which comprises kill switch molecular modification comprising (i) a cell death gene operatively associated with (ii) a first regulatory region comprising a first inducible promoter which is induced by exposure to blood or serum. In order for the synthetic microorganism to durably occupy a dermal or mucosal niche in the subject, the kill switch preferably should be silent (not expressed) in the absence of blood or serum.

[00427] In order to avoid “leaky expression” of the cell death gene, the synthetic microorganism may further comprise at least a second molecular modification (expression clamp) comprising (iii) an antitoxin gene specific for the cell death gene, wherein the antitoxin gene is operably associated with (iv) a second regulatory region comprising a second promoter which is active (e.g., constitutive) upon dermal or mucosal colonization or in a media (e.g., TSB), and preferably is downregulated by exposure to blood, serum or plasma.

[00428] The basal level of gene expression (the expression observed when cells are not exposed to blood or serum, e.g., in TSB (tryptic soy broth)) in the KS strain should ideally be very low because producing the toxin prior to contact with serum would kill or weaken the strain prematurely. Even moderate cell health impairment is unacceptable because: 1) escape mutations in the KS would accumulate (KS instability) – a known phenomenon that must be avoided, and/or; 2) the natural efficacy observed with our strain in preliminary trials could be reduced or lost. To understand if leaky expression is a problem, both the absolute level of baseline expression and the fold change in serum are being measured and closely considered in the selection of the optimal control region to drive the KS.

[00429] Awareness of leaky expression does not fix the problem and the reality is that even widely used “tightly controlled” rheostatic promoters such as P_{CUP1} and P_{Gal7} , and P_{Tet} -on/off variants produce measurable mRNA transcription in the absence of specific induction. In some embodiments, an “expression clamp” is employed in which the KS cassette contains not only the serum-responsive control region that drives toxin expression, but also encodes a “translation blocking” RNA under control of a *Staphylococcus aureus* promoter (P_{clfB} etc) that is normally strongly active in *Staphylococcus aureus* during colonization of the skin, and is downregulated in blood.

[00430] The *clfB* gene promoter (P_{clfB}) will be cloned to drive expression of the *sprA1**sprA1*_{AS} RNA and the cassette will be incorporated into the same expression module as is used for expression of the *sprA1* toxin from a serum-responsive promoter (eg, P_{isdB} , P_{hlga} etc). In this strain, serum/blood exposure activates the toxin (e.g., up to 350-fold or more) but not the antitoxin, and growth in TSB or on the skin activates antitoxin but not toxin. A representative diagram of an exemplary molecular modification of a synthetic strain is shown in FIG. 1.

[00431] An alternate approach to a synthetic microorganism: KO method

[00432] An alternative way to create a kill-switch-like phenotype in the synthetic microorganism is to disrupt ("knock-out") one or more genes that are required for survival in blood and/or for infection of organs but that are not required (or important) for growth in media or on the skin. In some embodiments, one or more, or two or more, of the 6 genes shown in Table 5 may be employed in the KO method.

[00433] Table 5: Candidates for gene knockout to create an attenuated strain:

Reference	Type of mutagenesis	Genes required for survival in blood or infection of organs	Reported gene function
Benton et al (2004) Large-Scale Identification of Genes Required for Full Virulence of <i>Staphylococcus aureus</i> . J. bact. 186(24): 8478–8489. DOI 10.1128/JB.186.24.8478–8489.2004	Transposon insertion	PycA; AspB; GabP. Mutation of these causes up to 1000-fold reduction in rate of organ infection in vivo	PycA: Pyruvate carboxylase
			AspB: Aspartate aminotransferase
			GabP: Gamma-aminobutyrate permease
Valentino et al (2014). Genes Contributing to <i>Staphylococcus aureus</i> Fitness in Abscess- and Infection-Related Ecologies. mBio5(5):e01729 - 14.doi:10.1128/mBio.01729-14.	Transposon insertion	Genes essential for in vitro survival in blood but not needed for growth in BHI liquid or agar: - SAOUHSC_01216 - SAOUHSC_00686 - SAOUHSC_00378	SAOUHSC_01216 : succinyl CoA-synthetase subunit b.
			SAOUHSC_00686: Unknown hypothetical protein
			SAOUHSC_00378: Unknown hypothetical protein

[00434] In one embodiment, a synthetic microorganism is provided comprising replacement of one or more of the genes in Table 5 with unmodified or expression-

clamped KS, using allelic exchange. This may further enhance the death rate of the synthetic microorganism in blood. Alternatively, the need to integrate two KSs is diminished by having one KO and one KS. In a further embodiment, a synthetic microorganism may comprise a combination of more than one KO that may have synergistic effects.

[00435] Kill Switch Regulatory Region

[00436] A synthetic microorganism comprising a kill switch is provided. The kill switch comprises a cell death gene operably linked to a regulatory region (RR) comprising an inducible promoter, as described herein.

[00437] Development of a synthetic microorganism involves identification and characterization of optimal regulatory regions (RRs) in order to drive kill switch genes; a list of serum responsive loci are chosen; RR's are identified; and Serum activation response is verified, and basal expression is investigated.

[00438] Identification and characterization of optimal regulatory regions to drive kill switch candidates.

[00439] This important phase of KS strain construction involves identifying genes that are strongly upregulated in response to human serum and/or whole heparinized blood. Once the genes are identified, their RR's, which contain the promoter and other upstream elements, are identified and annotated. In one approach, any known serum- and blood-responsive gene in *Staphylococcus aureus* may be employed that is known in the literature.

[00440] A RR includes the upstream regulatory sequences needed for activation (or repression) of mRNA transcription in response to stimuli. The motifs include "up" elements, -35, and -10 consensus elements, ribosome binding sites ("shine-dalgarno sequence") and "operator" sequences which bind protein factors that strongly influence transcription. In practice for eubacteria, harnessing a 200 bp region of DNA sequence upstream of the start codon is usually adequate to capture all of these elements. However, it is preferred to deliberately identify these sequences to ensure their inclusion.

[00441] Six *Staphylococcus aureus* genes that are strongly upregulated by exposure to human blood or serum are shown in Table 6.

[00442] Table 6: Identification of candidate RRs and serum or blood inducible promoters to drive kill switch components for driving the toxin.

Gene	Function	First author, year	Fold change in serum or blood	Time of exposure to blood or serum	SA strain used in study	Comments
<i>spa</i>	Staphylococcal Protein A; Ig binding; monocistronic gene	Malachowa 2011	~ 45 fold	90 min	USA300 and mu50	Wang 2004 predicts the monocistronic gene structure. Both experimental& computational evidence of this structure exist
<i>sir</i>	Sir ABC; iron transport	Malachowa 2011 and Wang 2004	81 fold in serum; 68-fold in blood (sirA; first ORF in operon)	30 to 120 minutes	USA300 and mu50	High induction at earliest timepoint. Experimental and predicted operon structure match
<i>ssr</i>	SsrABCD operon; Iron transport	Malachowa 2011 and Wang 2004	25-fold in serum; 15 fold in blood;	30 to 120 minute	USA300 and mu50	High induction at earliest timepoint. Experimental and predicted operon structure match
Gamma hemolysin <i>hlgA</i>	rbc lysis	Malachowa 2011;	~ 350-fold (Fig 4b)	90 min	USA300	Operon structure characterized by Cooney 1993
<i>sai-1</i> (seg 7 surface	29 kd cell surface	Wiltshire 2001	50-fold serum; 24-fold in	16h (O/N plating assay)	8325-4	Serum agar and solution phase assays, separate

protein). Also called <i>isdA</i>	protein; heme transporter;		blood. IsdB from the same operon is upregulated 240-fold in serum and 140-fold in blood			pubs. Serum was sufficient for induction in Wiltshire 2001 & Malachowa 2011.
<i>leuA</i>	2-isopropylmalate synthase	Malachowa 2011	-6 fold downreg. in TSB; 15 fold upreg in serum; 12 fold upreg in blood	30 to 120 min	USA300	Attractive b/c of downreg. in TSB but the fold upreg. in serum might be insufficient
SAUSA300_0119	Ornithine cyclodeaminase family protein	Malachowa 2011	50 fold upreg. in serum, 27 fold in blood; no upreg in TSB compared to time 0 in TSB	30 to 120 min	USA300	Different category of gene than above and also seemingly tightly regulated in TSB
<i>lrgA</i>	Murein hydrolase transporter	Malachowa 2011	-3.3 fold downreg in TSB; 12 fold upreg in serum; 17 fold upreg in blood	30 to 120 min	USA300	Attractive b/c it is down-regulated in TSB
<i>bioA</i>	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Malachowa 2011	107 fold upreg in serum; 56 fold upreg in blood; no reg in TSB	30 to 120 min	USA300	Attractive b/c very strong upreg and a lesser known metabolic gene

[00443] The full genes in each operon and the flanking sequences from strain BioPlx-01 are obtained from Genbank and annotated based on the literature plus known motif-identifying algorithms. Transcription terminators have been identified through a combination of published experiments and predictive tools.

[00444] Additional Literature evidence of expression of serum responsive promoters in TSB (or similar media) was investigated. For example, *spa* gene and *isdA* gene are disclosed in Ythier et al 2012, Molecular & Cellular Proteomics, 11:1123–1139, 2012. The *sirA* gene is disclosed in Dale et al, 2004 J Bacteriol 186(24) 8356-8362. The *sst* gene is disclosed in Morrissey et al. 2000. The *hlgA* gene is disclosed in Flack et al 2014, PNAS E2037-E2045. www.pnas.org/cgi/doi/10.1073/pnas.1322125111. The *leuA* gene is disclosed in Lei et al 2015, Virulence 6:1, 75-84.

[00445] Since these data come from many different strains and experimental systems, the entire collection may be assessed for expression in a single standardized assay system with quantitative gene expression measurements made by using real time PCR. Importantly, the basal "leaky" level of gene expression (the expression observed when cells are not exposed to blood or serum, e.g., in TSB) should be very low because producing the toxin prior to contact with serum would kill/weaken the BioPlx-XX strain (synthetic microorganism comprising a kill switch) prematurely. Even moderate cell health impairment is unacceptable because: 1) escape mutations in the KS would accumulate (KS instability) – a known phenomenon that must be avoided, and/or 2) the natural efficacy observed with BioPlx-01 could be reduced or lost. Thus, both the absolute level of baseline expression and the fold change in serum may be measured and closely considered in the selection of the optimal RRs to drive the KS. It is noted that *leuA* is downregulated in TSB (6-fold) and upregulated in serum (15-fold) making its RR particularly interesting candidate to control KS expression.

[00446] In some embodiments, the synthetic microorganism having a kill switch may further comprise an "expression clamp" in which the KS cassette contains not only the serum-responsive RR that drives toxin expression, but also encodes a "translation blocking" RNA antitoxin under control of a promoter that is normally active on the skin or nasal mucosa during colonization. The kill switch may encode an antitoxin that is capable of suppressing the negative effects of the cell death toxin gene.

[00447] In some embodiments, the synthetic microorganism is a *Staphylococcus aureus* having a molecular modification comprising a kill switch which further comprises an "expression clamp" in which the KS cassette contains not only the serum-responsive RR that drives toxin expression, but also encodes a "translation blocking" RNA antitoxin under control of a *Staphylococcus aureus* promoter (P_{clfB} etc.) that is normally active on the skin during colonization, for example, as shown in Table 7.

[00448] From those promoters listed on Table 6 plus real time PCR data, two or more RRs with the best mix of low basal expression and high response to serum/blood may be selected to drive KS expression. These RRs may be paired with 3 different KS genes as described herein, generating a panel of KS candidate strains for testing. The panel will include an "expression clamp" candidate as described next.

[00449] **Expression clamp to block toxin expression when the KS strain is on the skin or nasal epithelia**

[00450] The synthetic microorganism may comprise an expression clamp. Genes involved in *Staphylococcus aureus* colonization of human nares are shown in Table 7 may be employed as a second promoter for use in an expression clamp further comprising an antitoxin gene to block leaky toxin expression when the synthetic strain is colonized on skin or mucosal environments. The second promoter may be a constitutive promoter, such as a housekeeping gene. The second promoter may be preferably downregulated in the presence of blood or serum.

[00451] Table 7. Genes involved in *Staphylococcus aureus* colonization of human nares

Gene	Known or Putative role	Reference	Comments
<i>clfB</i> (Clumping factor B) (ClfB)	Adhesion	Wertheim HF, Walsh 2008; also Burian 2010	10 fold higher than Gyr in vivo; same high expression as <i>gyr</i> in vitro. Also, expression in rodent models and in humans is important for nasal colonization. It is

			expressed in exponential phase in vitro. Gene is downregulated 3-fold in human serum (Malachowa 2011)
autolysin (<i>sceD</i>) (exoprotein D)	Lytic transglycosylase	Stapleton MR, Horsburgh MJ 2007	expressed in exponential phase in vitro
<i>walKR</i> (virulence regulator)	essential master regulator of virulence	Burian 2010	In vivo expression at time zero and at year 1 is on par with <i>gyrA</i>
<i>atlA</i> (Major autolysin)	major autolysin; Bifunctional peptidoglycan hydrolase	Burian 2010	Similar characteristics as <i>walKR</i> but expression is higher (5 fold above <i>gyr</i>)
<i>oatA</i> (O-acetyltransferase A)	O-acetylation of peptidoglycan; renders <i>Staphylococcus aureus</i> cells resistant to lysozyme	Burian 2010	Similar to <i>WalKR</i>

[00452] In some embodiment, a synthetic microorganism is provided having a molecular modification comprising a kill switch and further comprising an expression clamp comprising an antitoxin gene driven by a second promoter that is normally active on the skin or nasal mucosa during colonization, optionally wherein the second promoter is selected from a gene selected from or derived from clumping factor B (*clfB*), autolysin (*sceD*; exoprotein D), *walKR* (virulence regulator), *atlA* (Major autolysin), and *oatA* (O-acetyltransferase A), as shown in Table 7. The constitutive second promoter may alternatively be selected from or derived from a housekeeping gene, for example, *gyrB*,

sigB, or *rho*, optionally wherein the second promoter comprises a nucleotide sequence of SEQ ID NO: 324, 325, or 326, respectively, or a substantially identical sequence.

[00453] The second promoter for use in the expression clamp may be selected from a gene identified in the target microorganism that has been recognized as being downregulated upon exposure to blood or serum.

[00454] The second promoter for use in an expression clamp molecular modification should be a constitutive promoter that is preferably downregulated upon exposure to blood or serum after a period of time, e.g., after 15 minutes, 30 minutes, 45 minutes, 90 minutes, 120 minutes, 180 minutes, 240 minutes, 360 minutes, or any time point in between, to decrease transcription and/or expression of the cell death gene, by at least 2-fold, 3-fold, 4-fold, 5-fold, or at least 10-fold, compared to transcription and/or expression in the absence of blood or serum.

[00455] The second promoter may be selected by a process comprising selecting a target microorganism, selecting one or more second promoter candidate genes in the target microorganism, growing the microorganism in a media, obtaining samples of the microorganism at $t=0$ min, adding serum or blood to the media, obtaining samples at $t=n$ minutes, where $n=1-240$ min or more, 15-180 min, or 30-120 min, performing RNA sequencing of the samples, and comparing RNA sequencing read numbers for candidate first promoter in samples obtained at $t=0$ min, and $t=n$ minutes after exposure to blood or serum for the candidate first promoter gene. Alternatively, samples obtained after $t=n$ minutes after exposure to blood or serum may be compared to $t=n$ minutes in media without blood or serum for the candidate second promoter. Candidate second promoters may be selected from those that exhibit downregulation by RNA sequencing after target cell growth at $t=n$ min in blood or serum, when compared to the candidate promoter in the target cell at $t=0$, or when compared to the candidate promoter in the target cell at $t=n$ in media without serum or blood.

[00456] The second promoter may be selected from or derived from a promoter candidate gene identified herein for potential use in an expression clamp in *Staphylococcus aureus* 502a that were found to be downregulated by at least 2-fold after exposure to serum for 30 minutes as determined by RNA sequencing as compared to $t=0$ including phosphoribosylglycinamide formyltransferase gene CH52_00525 (-4.30 fold), phosphoribosylaminoimidazole synthetase gene CH52_00530 (-4.27 fold),

amidophosphoribosyltransferase gene CH52_00535(-4.13 fold), phosphoribosylformylglycinamide synthase gene CH52_00540 (-4.04 fold), phosphoribosylformylglycinamide synthase gene CH52_00545 (-3.49 fold), phosphoribosylaminoimidazole-succinocarboxamide gene CH52_00555 (-3.34 fold), trehalose permease IIC gene CH52_03480 (-3.33 fold), DeoR family transcriptional regulator gene CH52_02275(-2.55 fold), phosphofructokinase gene CH52_02270 (-2.46 fold), and PTS fructose transporter subunit IIC gene CH52_02265 (-2.04 fold).

[00457] The second promoter may be selected from or derived from phosphoribosylglycinamide formyltransferase gene CH52_00525, trehalose permease IIC gene CH52_03480, DeoR family transcriptional regulator gene CH52_02275, phosphofructokinase gene CH52_02270, or PTS fructose transporter subunit IIC gene CH52_02265.

[00458] The second promoter may be a *P_{clfB}* (clumping factor B) gene; optionally wherein the second promoter comprises a nucleotide sequence of SEQ ID NO: 7, 117, 118, 129 or 130, or a substantially identical sequence.

[00459] In one specific example, one of the KS constructs (*sprA1*) is equipped with an expression clamp comprising an antitoxin (*sprA1_{AS}*) driven from the Clumping factor B (*clfB*) promoter. This promoter is one choice to drive the clamp because it is strongly expressed in TSB and during nasal/skin colonization (10 fold higher than the abundant housekeeping gene *gyrA*) (Burian 2010). This is directly relevant to manufacturing and use of the product, respectively. The Clumping factor B (*clfB*) promoter is also downregulated 3 fold in blood (Malachowa 2011), favoring clamp inactivity when. Complete inactivity in blood may not be needed because the serum-responsive promoters driving is so robustly activated in the blood.

[00460] The Clumping factor B (*clfB*) promoter is also stably expressed over at least 12 months during nasal colonization in humans and was also identified in rodent and *in vitro* models of colonization (Burian 2010).

[00461] In one example of an expression clamp, *clfB* is selected as a constitutive promoter for use in an expression clamp after confirmation of strong expression in TSB, and lower levels of expression in blood or serum (real time PCR), to determine its characteristics in target strain *Staphylococcus aureus* 502a. The *clfB* regulatory region is cloned to drive expression of the *sprA1* antisense (antitoxin) RNA (see Table 3, first

entry), and the cassette is incorporated into the same expression shuttle vector as is used for expression of the *sprA1* toxin gene from a serum-responsive promoter. It is desirable that the serum/blood exposure may strongly activate the toxin but not the antitoxin, and TSB or skin/nasal epithelial exposure activates antitoxin but not toxin. This concept may be applied to the other KS genes in Table 3 below. An alternative possibility for using the clamp is for the restriction enzyme KpnI (toxin) approach for which the antitoxin may be an RNA aptamer that was recently developed as a potent inhibitor of this enzyme (Mondragon, 2015) as a means of imparting metabolic stability to the aptamer.

[00462] Awareness of leaky expression does not fix the problem and the reality is that even widely used "tightly controlled" rheostatic promoters such as P_{CUP1} and P_{Gal7} , and P_{Tet} -on/off variants produce measurable mRNA transcription in the absence of specific induction.

[00463] The expression clamp comprises a second promoter operably linked to an antitoxin gene. For example, the antitoxin gene is specific for the cell death toxin gene in the kill switch in order to be effective. Under normal physiological conditions, the expression clamp acts to prevent leaky expression of the cell death gene. When exposed to blood or serum, the second promoter operably linked to the antitoxin is downregulated, allowing expression of the cell death gene.

[00464] The synthetic microorganism may contain an expression clamp comprising an antitoxin gene which is specific for silencing the cell death gene. The antitoxin may be selected or derived from any antitoxin specific for the cell death gene in the kill switch molecular modification that is known in the art. The antitoxin gene may encode an antisense RNA specific for the cell death gene or an antitoxin protein specific for the cell death gene.

[00465] The antitoxin gene may be a *sprA1* antitoxin gene, or *sprA1*(AS). The *sprA1* antitoxin gene may comprise a nucleotide sequence of TATAATTGAGATAA CGAAAATAAGTATTTACTTATACACCAATCCCCTCACTATTTGCGGTAGTGA GGGGATTT (SEQ ID NO: 311), or a substantially identical sequence, or CCCCTCACTA CCGCAAATAGTGAGGGGATTGGTGTATAAGTAAATACTTATTTTCGTTGT(S EQ ID NO: 273), or a substantially identical sequence.

[00466] The antitoxin gene may be a sprA2 antitoxin, or sprA2(AS), and may comprise a nucleotide sequence of
TATAATTAATTACATAATAAATTGAACATCTAAATACA
CCAAATCCCCTCACTACTGCCATAGTGAGGGGATTTATT (SEQ ID NO: 306),
or a substantially identical sequence; or
TATAATTAATTACATAATAAATTGAACATCTAAAT
ACACCAAATCCCCTCACTACTGCCATAGTGAGGGGATTTATTTAGGTGTTGG
TTA (SEQ ID NO: 312), or a substantially identical sequence.

[00467] The antitoxin gene may be a sprG antitoxin gene, also known as sprF, and may comprise a nucleotide sequence of (5'-3') ATATATAGAAAAAGGG
CAACATGCGCAAACATGTTACCCTAATGAG
CCCGTTAAAAAGACGGTGGCTATTTTAGATTAAAGATTAAATTAATAACCA
TTTAACCATCGAAACCAGCCAAAGTTAGCGATGGTTATTTTTT (SEQ ID NO:
307), or a substantially identical sequence. Pinel-Marie, Marie-Laure, Régine Brielle, and Brice Felden. "Dual toxic-peptide-coding *Staphylococcus aureus* RNA under antisense regulation targets host cells and bacterial rivals unequally." *Cell reports* 7.2 (2014): 424-435.

[00468] The antitoxin gene may be a yefM antitoxin gene which is specific for silencing yoeB toxin gene. The yefM antitoxin gene may comprise a nucleotide sequence of
MIITSPTEARKDFYQLLKNVNNNHEPIYISGNNNAENNAVIIGLEDWKSIIQETIYLE
STGTMDKVREREKDNSGTTNIDDIDWDNL (SEQ ID NO: 314), or a substantially identical nucleotide.

[00469] The antitoxin gene may be a lysostaphin antitoxin gene specific for a lysostaphin toxin gene. The lysostaphin antitoxin may comprise a nucleotide sequence of
TATAATTGAGATATGTTTCATGTGTTATTTACTTATACACCAATCCCCTCACT
ATTTGCGGTAGTGAGGGGATTTTTT (SEQ ID NO: 319), or a substantially identical nucleotide sequence.

[00470] The antitoxin gene may be a mazE antitoxin gene that targets mazF. The mazE toxin gene may comprise a nucleotide sequence of
ATGTTATCTTTTAGTCAAAAT

AGAAGTCATAGCTTAGAACAATCTTTAAAAGAAGGATATTCACAAATGGCT
GATTTAAATCTCTCCCTAGCGAACGAAGCTTTTCCGATAGAGTGTGAAGCA
TGCGATTGCAACGAAACATATTTATCTTCTAATTC (SEQ ID NO: 322), or a
substantially identical sequence.

[00471] The antitoxin gene may alternatively be designed as follows. In *Staphylococcus aureus*, there are two main methods used for gene silencing. In one style of gene silencing, which is exemplified by sprA1, antisense RNA binds to the 5' UTR of the targeted gene, blocking translation of the gene and causing premature mRNA degradation. Another style of gene silencing is used for genes that do not have a transcriptional terminator located close to the stop codon. Translation can be controlled for these genes by an antisense RNA that is complementary (~3-10 bases) to the 3' end of the targeted gene. The antisense RNA will bind to the mRNA transcript covering the sequence coding for the last couple codons and creating double stranded RNA which is then targeted for degradation by RNaseIII.

[00472] Since there are many examples of RNA silencing in *Staphylococcus aureus* that have been identified with demonstrated ability to control their target genes, these regions and sequences may be used as a base for designing the toxin/antitoxin cassettes. This approach requires only small changes in the DNA sequences.

[00473] In the present disclosure, the antitoxin for a cell death gene may be designed to involve antisense binding to 5'UTR of targeted gene. The toxin gene may be inserted into the PepA1 reading frame, and the 12bp in the endogenous sprA1 antisense is swapped out for a sequence homologous to 12bp towards the beginning of the heterologous toxin gene.

[00474] In one example, Holin inserted into the sprA1 location can be controlled by the antisense RNA fragment encoded by (12 bp Holin targeting sequence in **BOLD**)= TATA ATTGAGAT
AGTTTCATTAGCTATTTACTTATACACCAATCCCCTCA CTATTT
GCGGTAGTGA GGGGATTTTT (SEQ ID NO: 308).

[00475] In another example, 187-lysK inserted into the sprA1 location can be controlled by the antisense RNA fragment encoded by (12 bp 187-lysK targeting sequence in **BOLD**) TATAATTGAGAT **TTTAGGCAGTGC**

TATTTACTTATACACCAA TCCCCTCA CTATTTGCGGT
AGTGAGGGGATTTTT (SEQ ID NO: 309).

[00476] The antitoxin specific for the cell death gene may involve antisense binding to the 3' UTR of the toxin gene. This method involves inserting the heterologous toxin in the place of sprG in the genome of *Staphylococcus aureus*, and adding an additional lysine codon (AAA) before the final stop codon. The last 6 bases of the coding region (AAAAAA) plus the stop codon (TAA) overlap with the 3' region of the endogenous sprF antitoxin. When the sprF RNA is transcribed at a rate of 2.5 times greater than the heterologous toxin gene, it will form a duplex with the 3'UTR region of the toxin transcript, initiating degradation by RNaseIII and blocking the formation of a functional peptide. Since the 3' end of both of the heterologous toxins were manipulated in the same manner to overlap with the sprF sequence (adding the codon AAA in front of the TAA stop codon), which is also the same as the endogenous sprG 3' end, the sequence of the antitoxin will remain the same for all three of these toxin genes. For example, the sprG antitoxin gene (sprF) may comprise the nucleotide sequence ATATATAGAAAAA

GGGCAACATGCGCAAACATGTTACCCTAATGAGCCC
GTTAAAAAGACGGTGGCTATTTTAGATTAAAGATTAAATTAATAACCATTT
AACCATCGAAACCAGCCAAAGTTAGCGATGGTTATTTTTT (SEQ ID NO: 310).

[00477] The antitoxin gene may comprise a nucleotide sequence selected from any of SEQ ID NOs: 273, 306, 307, 308, 309, 310, 311, 312, 314, 319, or 322, or a substantially identical sequence thereof.

[00478] The antitoxin gene may or may not encode an antitoxin peptide. Wherein the synthetic microorganism is derived from a *Staphylococcus aureus* strain, the antitoxin peptide may be specific for the toxin peptide encoded by the cell death gene. For example, when the toxin gene is a *yoeB* toxin gene, e.g., encoding a toxin peptide comprising an amino acid sequence of SEQ ID NO: 316, the antitoxin gene may encode a yefM antitoxin protein comprising the amino acid sequence of MIITSPTEARKDFYQLLKNVNNNHEPI YISGNNAENNA
VIIGLEDWKSIIQETIYLESTGTMDKVREREKDNSGTTNIDDIDWDNL (SEQ ID NO: 314), or a substantially similar sequence. As another example, wherein the antitoxin gene is a mazF toxin gene, e.g., encoding a toxin peptide comprising an amino acid sequence of SEQ ID NO: 321, the antitoxin gene may be an mazE antitoxin

gene, e.g., encoding an antitoxin protein comprising an amino acid sequence of
 MLSFSQNRSHSLEQSLKEGYSQ
 MADLNLSLANEAFPIECEACDCNETYLSSNSTNE (SEQ ID NO: 323), or a
 substantially similar sequence.

[00479] Three KS candidate genes were selected as being of particular interest because they elicit cell death in 3 disparate ways. In some embodiments, the synthetic microorganism comprises one or more, two or more or each of *sprA1*, *kpnI* or *rsaE* to achieve maximal death rates as early data instruct. The *sprA1* mechanism of action is a loss of plasma membrane integrity/function by expression of a pore-forming peptide. the *kpnI* mechanism of action involves destruction of the *Staphylococcus aureus* genome with a restriction enzyme. The *rsaE* mechanism of action involves impairment of central metabolism including TCA cycle and tetrahydrofolate biosynthesis.

[00480] In some embodiments, the synthetic microorganism comprises regulatory region comprising a first promoter operably linked to a cell death gene, wherein the cell death gene encodes a toxin peptide or protein, and wherein the first promoter is upregulated upon exposure to blood or serum. The cell death gene may be a *sprA1* gene. *SprA1* encodes toxin peptide PepA1 as described in Sayed et al., 2012 JBC VOL. 287, NO. 52, pp. 43454–43463, December 21, 2012. PepA1 induces cell death by membrane permeabilization. PepA1 has amino acid sequence:
 MLIFVHIIAPVISGCAIAFFSYWLSRRNTK SEQ ID NO: 104. Related antimicrobial peptides include MMLIFVHIIAPVISGCAIAFFSYWLSRRNTK (SEQ ID NO: 105), AIAFFSYWLSRRNTK (SEQ ID NO: 106), IAFFSYWLSRRNTK (SEQ ID NO: 107), AFFSYWLSRRNTK (SEQ ID NO: 108), FFSYWLSRRNTK (SEQ ID NO: 109), FSYWLSRRNTK (SEQ ID NO: 110), SYWLSRRNTK (SEQ ID NO: 111), or YWLSRRNTK (SEQ ID NO: 112), as described in WO 2013/050590, which is incorporated herein by reference. The cell death gene may be an *sprA2* gene. The *sprA2* gene may encode a toxin
 MFNLLINIMTSALSGCLVAFFAHWLRTRNNKKGDK (SEQ ID NO: 305). The cell death gene may be a *Staphylococcus aureus* *yoeB* gene which may encode a *yoeB* toxin having the amino acid sequence of
 MSNYTVKIKNSAKSDLRKIKHSYLKKSFLIEIVETLKN
 D
 PYKITQSFEKLEPKYLERYSSRRINHQHRVVYTVDDRNKEVLILSAWSHYD (SEQ ID NO: 316), or a substantially similar sequence. The cell death gene may be a

Staphylococcus simulans gene which may encode a metallopeptidase toxin gene having an amino acid sequence of

MTHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVKAISSGKI
VEAGWSNYGGGNQIGLIENDGVHRQWYMHLKYNVKGVDYVKAGQIIGWSG
STGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGYGKAGGTVTPTPNTGWK
TNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDG
HVWVGYTGN SGQRIYLPVRTWNKSTNTLGVLWGTIK (SEQ ID NO: 318), or a

substantially similar sequence. The cell death gene may be a *mazF* toxin gene that encodes a *mazF* toxin comprising an amino acid sequence of

MIRRGDVYLADLSPVQGSEQGGVRPVVIIQNDTGKNYSPTVIVAAITGRINKAK
IPTHVEIEKKKYKLDKDSVILLEQIRTLDDKKRLKEKLTYLSDDKMKEVDNALMI
SLGLNAVAHQKN (SEQ ID NO: 321), or a substantially similar sequence.

[00481] The cell death gene may encode a toxin peptide or protein comprising an amino acid sequence of SEQ ID NO: 104, 105, 106, 107, 108, 109, 110, 111, 112, 285, 287, 289, 291, 305, 316, 318, or 321, or a substantially similar amino acid sequence. Preferably, the first promoter is silent, is not active, or is minimally active, in the absence of blood or serum.

[00482] PepA1 is a toxic pore forming peptide that causes *Staphylococcus aureus* death by altering essential cell membrane functions. Its natural role is unknown but speculated to be altruistic assistance to the *Staphylococcus aureus* population/culture by killing of cells that are adversely affected by environmental conditions. By over-expressing this gene a rapid and complete cell death occurs in the presence of serum. Of note, *sprA1* mRNA translation is repressed by an antisense RNA called *sprA11* (SprA1 antisense). The *cis-encoded* SprA1_{AS} RNA operates in *trans* to downregulate the *sprA1*-encoded peptide expression *in vivo*, as described in WO 2013/050590, which is incorporated herein by reference. The antisense RNA may in fact be a convenient safeguard to minimize "leaky" toxicity. It will be driven from a promoter that is expressed in *Staphylococcus aureus* on the human skin and nasal epithelia during colonization. Advantages of *sprA1* include the expression of a small peptide, having known structure and activity.

[00483] In a particular embodiment, a synthetic microorganism is provided comprising a first cell death gene *sprA1* operably linked to a first regulatory region comprising a blood and/or serum inducible first promoter comprising a nucleotide

sequence of any one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, or 163. The first promoter may be upregulated greater than 5-fold, greater than 10-fold, greater than 50-fold, greater than 100-fold, greater than 300-fold, or greater than 600-fold after 15, 30, 45, 60, 90, 120, 180 or 240 minutes of incubation in blood or serum. The first promoter may be upregulated greater than 5-fold after 90 minutes of incubation in serum and may be selected from *fhuA*, *fhuB*, *isdI*, *isdA*, *srtB*, *isdG*, *sbnE*, *sbnA*, *sbnC*, and *isdB*. The first promoter may be upregulated greater than 100-fold after 90 minutes of incubation in serum and may be selected from *isdA*, *srtB*, *isdG*, *sbnE*, *sbnA*, *sbnC*, and *isdB*.

[00484] The cell death gene may encode an antimicrobial peptide comprising an amino acid sequence of SEQ ID NO: 104, 105, 106, 107, 108, 109, 110, 111, 112, 285, 287, 289, 291, 305, 316, 318, or 321, or a substantially similar amino acid sequence thereof.

[00485] The cell death gene may be selected from any known *Staphylococcus* spp. toxin gene. The cell death gene may be selected from a *sprA1* toxin gene, *sprA2* toxin gene, *187-lysK* toxin gene, holin toxin gene, *sprG* toxin gene, *yoeB* toxin gene, lysostaphin toxin gene, metallopeptidase toxin gene, or *mazF* toxin gene, or a substantially identical toxin gene. The toxin gene may comprise a nucleotide sequence of SEQ ID NO: 274, 275, 284, 286, 288, 290, 304, 315, 317, or 320, or a substantially identical nucleotide sequence thereof.

[00486] The cell death gene may be *sprA1* which encodes the antimicrobial peptide PepA1. In some embodiments, the synthetic microorganism further comprises an antitoxin gene *SprA1-AS* operably linked to a second regulatory region comprising a second promoter comprising a nucleotide sequence of *clfB* comprising a nucleotide sequence of SEQ ID NO: 7, 117, 118, 129 or 130, or a substantially identical sequence.

[00487] In some embodiments, the synthetic microorganism comprises a restriction enzyme KpnI (*Klebsiella pneumoniae*) gene. KpnI protects bacterial genomes against invasion by foreign DNA. High-level expression of (eg) 6-bp recognition restriction enzyme KpnI will efficiently cleave the *Staphylococcus aureus* genome. In some embodiments, the expression vector (below) will be engineered to lack cleavage recognition sites by (eg) adjustment of codon usage. The 6-base recognition sequence

occurs once every 4096 bp, cutting the 2.8 MB genome of *Staphylococcus aureus* into ~684 fragments. KpnI has the advantage of rapid activity. In some embodiments, "leaky" expression problem may be managed by expressing an RNA aptamer as the clamp as described above for *sprA1*.

[00488] In some embodiments, the synthetic microorganism comprises a *rsaE* gene. The *rsaE* gene is a small RNA (93 nt) that coordinately inhibits 2 different metabolic pathways by targeting translation initiation of certain housekeeping mRNAs encoding enzymes of THF biosynthesis pathway and citric acid cycle; high-level expression is toxic. By over-expressing RseE growth impairment occurs due to inhibition of essential housekeeping enzymes. This occurs by binding to the Opp3A and OppB mRNAs in the ribosome-binding site and start codon region, preventing translation. Both genes encode components of the ABC peptide transporter system and affect the supply of essential nitrogen/amino acids in the cell, impairing central biochemical metabolism directly and indirectly. Advantages include severe growth inhibition (10,000 fold over empty vector controls), and efficient multifunctionality because a single sRNA impairs expression of multiple essential biochemical pathways. Geissman et al. 2009 and Bohn et al. 2010 report on the natural function of RsaE.

[00489] Creation of a panel of serum-activated kill switch (KS) plasmid candidates for expression in *Staphylococcus aureus* is performed wherein serum responsive RRs are sub-cloned to *Staphylococcus aureus* shuttle vectors; cell death genes are inserted downstream of RRs, and sequenced; feasibility of leaky expression repressor "expression clamp" is tested; and candidate strains are completed and evaluated to select lead candidate(s) that exhibit rapid and complete death, and good baseline viability.

[00490] Chromosomal integration of optimal kill switch candidates is important for long-term stable expression. In addition, comparison of death rate extent and stability of strains *in vitro* is performed. Insertion of up to 3 optimal kill switch cassettes alone and in 3 combinations of two, for a total of up to 6 strains is performed. This achievement may require a multistep cloning in *E. coli* to build the constructs. For example, *E. coli* strain DC10B may be employed. DC10B is an *E. coli* strain that is only DCM minus (BEI product number NR-49804). This is one way to generate DNA that can be readily transfected into most *Staphylococcus aureus* strains. To this end, stable integrants are obtained, and plasmid vector is excised during counter selection. The rate and extent of serum-induced cell death is confirmed and characterized, and genetic stability is

determined for all 6 strains. A non-human functional test of preferred KS strain candidates is performed including a functional test of strain death *in vivo*; and a functional test of colonization-skin discs.

[00491] In some embodiments, a method for preparing a synthetic *Staphylococcus aureus* strain from BioPlx-01 is provided comprising (1) producing a shuttle vector pCN51 in mid-scale in *E. coli*, (2) cloning cell death genes into pCN51 in *E. coli* under Cd-inducible promoter P_{cad} , (3) replacing P_{cad} with serum-responsive promoters and optionally inserting expression clamp, (4) verifying constructs by sequencing KS cassettes, (5) electroporating into *Staphylococcus aureus* RN4220 and selecting transformants on erythromycin plates (this strain is restriction minus and generates the right methylation pattern to survive in BioPlx-01), (6) preparing plasmid from RN4220 and restriction digest to confirm identification, (7) electroporating plasmids into BioPlx-01 and select on erythromycin plates, and (8) isolating strains. Strains produced in this fashion are ready for performance testing and serum experimentation. The method is further described in detail herein.

[00492] In some embodiments, a method for performance testing a synthetic *Staphylococcus aureus* strain from BioPlx-01 is provided comprising (1) growing in TSB plus antibiotic as selective pressure for plasmid, (2) comparing growth to WT BioPlx-01 optionally generating a growth curve, (3a) for Cd-promoter variants, washing and shifting cells to Cd-medium (control is BioPlx-01 containing empty vector with no cell death gene) – or – (3b) for KS variants, washing and shifting cells to serum (control is WT BioPlx-01 containing empty vector with no cell death gene), and (4) monitoring growth using OD_{630 nm} with plate reader, optionally for extended period with monitoring for escape mutants. For whole blood test, the method is only performed on preferred candidates and using colony forming units (CFUs) on TSA as death readout. If colonies form on kill switch bearing strains after they have been exposed to blood, the plasmid should be sequenced to check for mutations. If there are escape mutants, shuttle plasmid out to *E. coli* and sequence whole plasmid.

[00493] Method for creation of serum-activated kill switch (KS) plasmid candidates for expression in *Staphylococcus aureus* (SA)

[00494] Methods are provided for evaluation of cell death induction comprises recombinant construction of the synthetic microorganism comprising cloning the genes

into an *E. coli*-SA shuttle vector and transfecting this vector into BioPlx-01 for evaluation.

[00495] Step 1: Request Shuttle Vector PCN51

[00496] A commercially available shuttle vector is obtained such as PCN51 (available through BEI) is one excellent choice as it contains: i) a cadmium-inducible promoter that can be used in positive control strains to prove the toxins are expressed and functional; ii) a universal Transcription terminator (TT) that will apply to all of our constructs; and, iii) well-established replicons for *E. coli* and *Staphylococcus aureus*. A schematic of commercially available shuttle vector pCN51 (BEI cat # NR-46149) is shown in FIG. 2. Genetic elements shown of pCN51 shuttle plasmid are shown in Table 8.

[00497] Table 8. Elements of pCN51 Shuttle Vector

Shuttle Plasmid pCN51 (BEI cat # NR-46149)	
Element	Purpose
pT181cop-WT repC	SA replication machinery
ermC	erythromycin resistance
Amp	beta-lactamase; confers resistance to ampicillin in <i>E. coli</i>
ColE1 Ori	Origin of replication for <i>E. coli</i>
Pcad-cadC	Cadmium-inducible promoter
MCS (black box)	Multiple Cloning Site; unique sites for cloning our KS.
TT	blaZ transcription terminator

[00498] Promoter sequences (7) used in development are shown below, the base pair numbers in *leuA*, *hlgA* and Cadmium promoters correspond to pCN51 vector location.

[00499] 1. *leuA* promoter (P_{leuA}) sequence between restriction sites SphI and PstI (underlined) amplified from genomic BioPlx-01 (502a) DNA.

SphI
GCATGCGAAA CAGATTATCT

5501 ATTCAAAGTT AATTGTAAGA AAATTTAAAA TATTTGTTGA CATACTAAAG
 5551 CAGATATAGT AAATTAAATT TATCAAATTT TTAGACAATT CTAACATATTA
 5601 AAGTGATATA TACCATTAC GGAAGGAGTA TAATAAAATG CTTAATCAAT
 5651 ATACTGAACA TCAACCGACA ACTTCAAATA TTATTATTTT ATTATACTCT
 5701 TTAGGACTCG AACGTTAGTA AATATTTACT AAACGCTTTA AGTCCTATTT
 5751 CTGTTTGAAT GGGACTTGTA AACGTCCCAA TAATATTGGG ACGTTTTTTT
 5801 ATGTTTTATC TTTCAATTAC TTATTTTAT TACTATAAAA CATGATTAAT
 5851 CATTAAAATT TACGGGGGAA TTTACTCTGC AG SEQ ID NO:114

PstI

[00500] 2. *hlgA* promoter (P_{hlgA}) sequence between restriction sites SphI and PstI amplified from genomic BioPlx-01 (502a) DNA.

SphI

GCATGC AAACTATTGC

5501 GAAATCCATT CCTCTTCCAC TACAAGCACC ATAATTAAAC AACAAATCAA
 5551 TAGAATAAGA CTTGCAAAAC ATAGTTATGT CGCTATATAA ACGCCTGCGA
 5601 CCAATAAATC TTTTAAACAT AACATAATGC AAAAACATCA TTTAACAATG
 5651 CTAAAAATGT CTCTTCAATA CATGTTGATA GTAATTAACT TTTAACGAAC
 5701 AGTTAATTCG AAAACGCTTA CAAATGGATT ATTATATATA TGAACCTAAA
 5751 ATTAAATAGA AAGAAAGTGA TTTCTCTGCA G SEQ ID NO: 115

PstI

[00501] 3. Cadmium promoter (P_{cad}) sequence between restriction sites SphI and PstI. This promoter is used for controls and is part of the original pCN51 vector from BEI Resources (<https://www.beiresources.org/>).

SphI

GCATGCGCAC TTATTCAAGT

5501 GTATTTTTTA ATAAATTATT TTACTTATTG AAATGTATTA TTTTCTAATG
 5551 TCATACCCTG GTCAAAACCG TTCGTTTTTG AGACTAGAAT TTTATGCCCT
 5601 ACTTACTTCT TTTATTTTCA TTCAAATATT TGCTTGCATG ATGAGTCGAA
 5651 AATGGTTATA ATACACTCAA ATAAATATTT GAATGAAGAT GGGATGATAA
 5701 TATGAAAAAG AAAGATACTT GTGAAATTTT TTGTTATGAC GAAGAAAAGG
 5751 TTAATCGAAT ACAAGGGGAT TTACAAACAG TTGATATTTT TGGTGTTAGC
 5801 CAAATTTTAA AGGCTATTGC CGATGAAAAT AGAGCAAAAA TTAATTACGC
 5851 TCTGTGTCAG GATGAAGAGT TGTGTGTTTG TGATATAGCA AATATCTTAG
 5901 GTGTTACGAT AGCAAATGCA TCTCATCATT TACGTACGCT TTATAAGCAA
 5951 GGGGTGGTCA ACTTTAGAAA AGAAGGAAAA CTAGCTTTAT ATTCTTTAGG
 6001 TGATGAACAT ATCAGGCAGA TAATGATGAT CGCCCTAGCA CATAAGAAAG
 6051 AAGTGAAGGT CAATGTCTGA ACCTGCAG SEQ ID NO: 116

PstI

[00502] 4. *clfB* promoter (P_{clfB}) to drive the antisense regulatory RNA *sprAI*_{AS}. This is the forward sequence with EcoRI and BamHI sites. This sequence is put in reverse to drive the *sprAI*_{AS} to potentially act as a clamp to keep the *sprAI* gene regulated in the absence of blood. Underlined represents EcoRI and BamHI sites, respectively.

EcoRI

GAATTCAGGTGATGAAAAATTTAGAACTTCTAAGTTTTTGAAAAGTAAAAAATTTGTAATAGTGTAAGAA
TAGTATATTGATTTTTGCTAGTTAACAGAAAATTTTAAGTTATATAAATAGGAAGAAAACAAATTTTACG
TAATTTTTTTTCGAAAAGCAATTGATATAATTCTTATTTTCATTATACAATTTAGACTAATCTAGAAATTGA
AATGGAGTAATATTTGGATCCSEQ ID NO: 117

[00503] P_{clfB} as it is cloned in pCN51 vector with EcoRI and BamHI reversed.

BamHI

GGATCCAAATATTACTCCATTTCAATTTCTAGATTAGTCTAAATTGTATAATGAAATAAGAATTATATCA
ATTGCTTTTCGAAAAAATTACGTAAAATTTGTTTTCTTCTATTTATATAACTTAAATTTTCTGTAA
CTAGCAAAAATCAATATACTATTTTTTACACTATTACAAATTTTTTACTTTTCAAAAACCTAGAAGTTCTA
AATTTTTTCATCACCTGAATTC

SEQ ID NO: 118

[00504] 5. The *sirA* promoter (P_{sirA}) as found in the NCBI 502a complete genome. This sequence was taken 300 base pairs upstream of the *sirA* start codon as shown underlined below.

ttgaaagatttacttttatatatgaagagactggattaaatacttttattgacgtaaaaattcacttttgaaccgttca
atatcttgccgattttatataacagctacaaataaaatataacagtttgattttacagcctcggtaaatcgctcttgaca
aacaaaaatttggctatcacacatttgcaacgtcttaacaagtcattctataaacatttctaaatatttaacattact
tatgcgtcatttattgctaaaattattgtattaaaatatacatagaattgatgggatatcATG SEQ ID NO: 119

[00505] 6. . The *sstA* promoter (P_{sstA}) as found in the NCBI 502a complete genome. This sequence was taken 300 base pairs upstream of the *sstA* start codon as shown underlined below.

acgaaaaattaattaacatcgcatgtttattactgcaactattacagcattggtagtggtgactgttggaaacattaccg
ttcttaggactagtaatacacaatattatttcaatttatcgaggtgatcattgaaaaatgctatccctcatagcatgat
gttaggtgccatctttgtatttttctgatatagttggcagaattgtgtttatccatagaataaatattggtttaa
caataggtgtatttgaacaatcatttccttattcttcttatgaaaggtaggaaaaattATG SEQ ID NO: 120

[00506] 7. The *isdA* promoter (P_{isdA}). This sequence was taken 300 base pairs upstream of the SstA start site as shown underlined below from the NCBI 502a complete genome.

CTATCTGCGGCATTTGCAGAATTACTGAATGTCGCGATGATGATAATTAACGCTAAAATCGT
TGTATTAAAACTTTTAAAAATATTTTTCAAAACATAATCCTCCTTTTTATGATTGCTTTTAAG
TCTTTAGTAAATCATAAATAATAATGATTATCATTGTCAATATTTATTTTATAATCAATTTA
TTATTGTTATACGGAAATAGATGTGCTAGTATAATTGATAACCATTATCAATTGCAATGGTT
AATCATCTCATATAACAACACATAATTTGTATCCTTAGGAGGAAAACAACATG SEQ ID
NO: 121.

[00507] In some embodiments, a plasmid, vector, or synthetic microorganism is provided comprising a molecular modification comprising a cell death gene operably linked to an inducible blood or serum responsive first promoter comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, and 163, or a substantially identical nucleotide sequence. In some embodiments, the molecular modification further comprises an expression clamp comprising an antitoxin gene operably linked to a second promoter comprising a nucleotide sequence selected from SEQ ID NO: 7, 117, 118, 129 or 130.

[00508] Step 2: Cloning best two serum-responsive RRs into the shuttle vector (*E. coli* host)

[00509] Cloning of candidate serum-responsive RRs into the shuttle vector (*E. coli* host) comprises: (a) PCR amplification of the best two preferred serum-responsive RRs from BioPlx-01 genomic DNA (gDNA); and (b) replacing the Cadmium-inducible promoter with these RR fragments in pCN51 to create two new plasmids (RR1 and RR2), and (3) selecting clones in *E. coli* DH10B (or DH5 alpha) and sequencing of insertions.

[00510] The following KS genes are obtained from *Staphylococcus aureus* gDNA or by *de novo* synthesis: (i) *sprA1/sprA1_{AS}*: synthetic; (ii) RsaE: *Staphylococcus aureus* genomic DNA. And (iii) KpnI: synthetic. For genes amplified from gDNA, PCR primers are used with relevant restriction enzymes for cloning. For synthetic genes, the cloning sites will be included at synthesis and any undesirable sites removed during construction. For example, KpnI sites will be removed from the *kpnI* cassette to prevent auto-digestion. The KS genes are inserted downstream of serum-responsive RRs in plasmids

RR1 and RR2, generating all constructs listed below. Insert the KS genes downstream of Cd-inducible promoter in pCN51 to create positive control constructs. See additional relevant sequences and primer sequences provided herein useful for these steps, for example, Tables 2, 3 and 4. Sequencing of promoters and inserts of all constructs is performed to ensure that mutations have not accumulated in the construction process

[00511] A list of Plasmid constructs to be produced is shown below. All but 2, 4, 8 and 11 will be transfected into *Staphylococcus aureus*.

- [00512] 1. Cd-inducible promoter-*sprA1*
- [00513] 2. Cd-inducible promoter-reverse orientation *sprA1*
- [00514] 3. Serum responsive RR1- *sprA1*
- [00515] 4. Serum responsive RR1-reverse orientation *sprA1*
- [00516] 5. Serum responsive RR1- *sprA1* + P_{clfB}-*sprA1*_{AS}
- [00517] 6. Serum responsive RR2- *sprA1*
- [00518] 7. Serum responsive RR1-*rsaE*
- [00519] 8. Serum responsive RR1- *rsaE* -reverse orientation
- [00520] 9. Serum responsive RR2- *rsaE*
- [00521] 10. Serum responsive RR1-*kpnI*
- [00522] 11. Serum responsive RR1- *kpnI* reverse orientation
- [00523] 12. Serum responsive RR2- *kpnI*

[00524] The reverse orientation constructs are being created in the process, because if a cell death gene has some basal toxicity even in growth medium, it may not be possible to obtain the forward orientation construct. Such a negative result is not conclusive unless the reverse orientation construct is readily obtained in side-by-side fashion.

[00525] Step 3: Transfect plasmids into intermediate *Staphylococcus aureus* RN4220 (to obtain correct DNA methylation pattern). There is no need to transfect reverse orientation constructs; but transfection of pCN51 empty vector is performed as follows:

- A. Electroporate into RN4220;
- B. Select transformants on plates containing erythromycin; and
- C. Isolate and confirm plasmid ID with restriction digests.

[00526] Step 4: Transfect into BioPlx-01

- A. Electroporate plasmids from step 3C into competent BioPlx-01;
- B. Select transformants by erythromycin resistance; and
- C. Isolate and confirm plasmid ID with restriction digests; save stocks of 9 strains.

[00527] Step 5: Test KS expression and extent and rate of death in response to serum and blood exposure

- A. Qualitative test of expression of kill genes with real time PCR pre- and post-blood/serum exposure. This will: i) confirm the strain construction; ii) correlate onset of toxin production with onset of death, and iii) determine promoter "leakiness" in the context of the KS;
- B. Cell death induction curves in serum/blood compared to TSB (killing extent and kinetics by CFU); and
- C. Simple growth rate comparison of BioPlx-01 containing empty vector vs. BioPlx-01 with the KS plasmids.

[00528] Step 6: Measure the rate of KS mutation

[00529] Count colonies that grow on serum or blood agar plates and/or in serum containing liquid media over several hundred generations via serial passaging. Determine if mutation rate is acceptable. It has been reported that the rate of functional KS loss is 10^{-6} for one copy of a KS gene, but as low as 10^{-10} for two copies of the same or different KS genes from two different promoters (Knudsen 1995; reporting on actual mutation rate assay measurements).

[00530] Step 7: Analysis and interpretation

[00531] The best KS strain(s) are those with unaffected growth rates (and colonization potential); and that show rapid and complete death in response to blood and/or serum; and that have stable molecular modifications.

[00532] Step 8: Determine need for inserting multiple KS cassettes

[00533] If the molecular stability of one KS is deemed inadequate, a second and different functional KS from the list of 9 candidates (if another functional one exists) will

be added to the plasmid and a re-test of killing and stability will be performed. A dramatic improvement in KS stability is anticipated on the basis of Knudsen 1995 and theoretical calculations.

[00534] Method for chromosomal integration of optimal kill switch(es), for long-term stable expression

[00535] The optimal serum/blood responsive KS construct(s) will be integrated into the chromosome precisely at a pre-selected location known to tolerate insertions without notably altering the cell's biology.

[00536] Step 1: Obtain an Integrative vector for use in *Staphylococcus aureus*.

[00537] After careful consideration to the optimal integrative vector, plasmids pKOR1 or pIMAY may be employed because they provide the ability to choose the integration site, allowing us to avoid perturbing biologically critical regions of the genome that can occur with other methods. Both vectors possess a convenient means for counter-selection (*secY*) so that the plasmid backbone and its markers can be excised from the genome after the KS has been integrated. A genetic map of pKOR1 is shown in FIG. 5A and the features are described in Bae et al. 2006 Plasmid 55, pp. 58-63, and briefly described in Table 9. An advantage of pKOR is the ability to clone inserts without the limits of specific restriction enzymes.

[00538] Table 9. Purpose of elements in pKOR integrative plasmid

Integrative Plasmid pKOR	
Element	Purpose
AmpR	beta-lactamase; confers resistance to ampicillin in <i>E. coli</i> (but not in <i>Staphylococcus aureus</i>)
Ori (-)	<i>E. coli</i> origin of replication
Attp1 and 2	Recombine with AttB elements of DNA inserts
CcdB	<i>E. coli</i> gyrase inhibitor protein; growth of cells containing non-recombinant plasmid are inhibited by this protein
Cat- and Cat +	Chloramphenicol resistance genes for use in gram neg and gram + bacteria respectively

SecY570	570 nt encoding essential N terminus of secY; its antisense is expressed from the ATc-inducible pxyl/tetO promoter; growth in the presence of Atc means the plasmid backbone has been lost
RepF	Replication gene for <i>Staphylococcus aureus</i>

[00539] A Genetic map of pIMAY is shown in FIG. 5B from Monk, IR et al., mBio 2012; doi:10.1128/mBio.00277-11. FIG. 12A-12C shows nucleotide sequence (SEQ ID NO: 131) of pIMAY Integrative Plasmid. (accession number JQ62198). The *E. coli*/staphylococcal temperature-sensitive plasmid pIMAYz comprises the low-copy-number *E. coli* origin of replication (p15A), an origin of transfer for conjugation (*oriT*), the pBluescript multiple cloning site (MCS), and the highly expressed *cat* gene (*Phelp-cat*) derived from pIMC. The temperature-sensitive replicon for Gram-positive bacteria (*repBCAD*) and the anhydrotetracycline-inducible antisense *secY* region (*anti-secY*) may be amplified from pVE6007 and pKOR1, respectively. The restriction sites listed are unique. Primers (IM151/152) bind external to the MCS of pIMAY and are used to screen clones in *E. coli* (amplify 283 bp without a cloned insert) and to determine the presence of a replicating plasmid in staphylococci. Advantages of pIMAYz are smaller size, blue white screening, and a lower nonpermissive temperature, which has been reported to avoid mutations that can occur in the integration process. Thus, the plasmid may be made by de novo gene synthesis at a contract vendor firm.

[00540] Step 2. Review selectable markers in BioPlx-01.

[00541] BioPlx-01 is sensitive to ampicillin (50 µg/mL and 100 µg/mL), chloramphenicol (10 µg/mL), and erythromycin (Drury 1965). In one embodiment, the chloramphenicol (*cat*⁺) gene is used to select for transformants on chloramphenicol plates during the integration process.

[00542] Step 3. Generate the DNA fragment to be integrated.

[00543] Prepare a plasmid in shuttle vector pTK1 that contains the following elements in tandem: [aTTB2]-[1 Kb of sequence upstream of target region to be replaced]-[KS cassette-AmpR]-[1 Kb of sequence downstream of target region] ATTB1 according to a modification of Bae et al., 2006. Drop the fragment out of this plasmid with restriction enzymes and isolate it. The "KS cassette" may actually be one or two copies of a KS, pending the outcome of genetic stability testing.

[00544] Step 4. Insert KS cassette(s) to pKOR plasmid.

[00545] Perform *in vitro* recombination of the fragment from step 3 with the plasmid PKOR1 and then transfect the recombination mixture into DH5 alpha and obtain desired plasmid construct by standard screening methods in *E. coli*, using restriction mapping to verify construction.

[00546] Step 5. Obtain the KS strain-containing integration plasmid, in BioPlx-01

[00547] Electroporate the plasmid into RN4220; isolate plasmid DNA from the thus transfected RN4220, and electroporate this DNA into BioPlx-01 and select transformants on TSA plates containing chloramphenicol (10 µg/mL).

[00548] Step 6. Plasmid integration to chromosome.

[00549] Shift the strains to the non-permissive temperature (43°C) to promote plasmid integration to the target site, and select a colony on a chloramphenicol plate (10 µg/mL).

[00550] Step 7. Counter selection to evict plasmid backbone

[00551] Grow the colony isolate from step 6 at the permissive temperature (30°C) to favor plasmid excision and plate on 2 µg/mL and 3 µg/mL anhydrotetracycline (aTc) agar to obtain colonies in which the target gene has integrated and the plasmid has been excised and lost (the counterselection step). Any colonies that grow on plates containing ≥ 2 µg/mL aTc do not contain the plasmid because the plasmid backbone contains the lethal aTc-derepressible SecY antisense gene.

[00552] Step 8. Confirm integrated allele sequence

[00553] Isolate genomic DNA from the KS strain and confirm the knock-in cassette and flanking structure by PCR (and sequencing of the PCR amplicon).

[00554] Step 9. Check serum-induced cell death

[00555] Once confirmed, conduct cell death rate assays by growing the cells first in TSB, then shifting to human blood or serum and determining the rate of death by CFU plating assays in TSA (10 days).

[00556] Step 10. Verify expression of KS mRNA

[00557] Confirm expression changes of the target gene in blood, serum, and in TSB.

[00558] Step 11. Prepare frozen banks

[00559] Animal studies may be performed with synthetic microorganisms BioPlx-XX created by these methods. *In vivo* functional studies to test kill switch strain function may be performed. Possible studies include a mouse study to show difference in pathogenicity of intravenous or intraperitoneal injection of wt BioPlx-01 vs. KS strain. An *in vitro* skin colonization test may also be performed. Additional tests may include, in mouse: LD₅₀ test, BioPlx-01 vs. BioPlx-XX is performed. As another example, in rat or other: colonization test, BioPlx-01 vs. BioPlx-XX is performed.

[00560] **CRISPR-Cas induced homology directed repair to direct insertion of optimal kill switch candidates for long term stable expression**

[00561] In some embodiments, a method for preparing a synthetic *Staphylococcus aureus* strain from BioPlx-01 is provided comprising use of CRISPR-Cas induced homology directed repair to direct insertion of optimal KS candidates for long-term stable expression. In some embodiments, a method for preparing a synthetic *Staphylococcus aureus* strain from BioPlx-01 is provided comprising (1) obtaining competent cells, (2) design and testing of CRISPR guide RNA (gRNA) sequences and simultaneously testing pCasSA, (3) designing and testing homology dependent repair templates using a fluorescent reporter controlled by a constitutive reporter, (4) checking KS promoters with fluorescent reporter, (5) inserting KS into BioPlx-01 and verifying incorporation, and (6) testing for efficacy and longevity. Optionally, inserting additional KS cassettes in alternative locations within BioPlx-01 genome is performed.

[00562] FIG. 10 shows cassette for integration via CRISPR and layout of the pCasSA vector. *Pcap1A* is a constitutive promoter controlling gRNA transcription. Target seq is targeting sequence, for example, with 10 possible cutting targets (1.1, 1.2 etc.). gRNA is single-strand guide RNA (provides structural component). *Xba*I and *Xho*I are two restriction sites used to add the homology arms (HAs) to the pCasSA vector. HAs are homology arms to use as templates for homology directed repair (200 – 1000 bp). *P_{rpsL}*-mCherry is a constitutive promoter controlling the “optimized” mCherry. *P_{rpsL}*-Cas9 is a constitutive promoter controlling Cas9 protein expression.

[00563] FIG. 11 shows vectors for various uses in the present disclosure. A is a vector used for promoter screen with fluorescence using pCN51. B is a vector for promoter screen with cell death gene. C is a vector for chromosomal integration using CRISPR. D is a vector for chromosomal integration using homologous recombination. L & R HA: homology arms to genomic target locus, CRISPR targeting: RNA guide to genomic locus, mCherry: fluorescent reporter protein, Cas9 protein: CRISPR endonuclease, kanR: kanamycin resistance, oriT: origin of transfer (for integration), and SmaI: representative kill gene (restriction endonuclease).

[00564] Administration and Compositions

[00565] In some embodiments, compositions are provided comprising a synthetic microorganism and an excipient, or carrier. The compositions can be administered in any method suitable to their particular immunogenic or biologically or immunologically reactive characteristics, including oral, intravenous, buccal, nasal, mucosal, dermal or other method, within an appropriate carrier matrix. In one embodiment, compositions are provided for topical administration to a dermal site, and/or a mucosal site in a subject. Another specific embodiment involves the oral administration of the composition of the disclosure.

[00566] In some embodiments, the replacing step comprises topically administering of the synthetic strain to the dermal or mucosal at least one host subject site and optionally adjacent areas in the subject no more than one, no more than two, or no more than three times. The administration may include initial topical application of a composition comprising at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , or at least 10^{10} CFU of the synthetic strain and a pharmaceutically acceptable carrier to the at least one host site in the subject. The initial replacing step may be performed within 12 hours, 24 hours, 36 hours, 48 hours, 72 hours, 4 days, 5 days, 6 days, 7 days, 8 days, or 9 days of the final suppressing step.

[00567] The composition comprising a synthetic microorganism may be administered to the dermal and/or mucosal at least one site in the subject, and optionally adjacent sites at least once, for example, from one to 30 times, one to 20 times, one to ten times, one to six times, one to five times, one to four times, one to three times, or one to two times, or no more than once, twice, three times, 4 times, 5 times, 6 times, 8 times per month, 10 times, or no more than 12 times per month. Subsequent administration of

the composition may occur after a period of, for example, one to 30 days, two to 20 days, three to 15 days, or four to 10 days after the first administration.

[00568] Colonization of the synthetic microorganism may be promoted in the subject by administering a composition comprising a promoting agent selected from a nutrient, prebiotic, stabilizing agent, humectant, and/or probiotic bacterial species. The promoting agent may be administered to a subject in a separate promoting agent composition or may be added to the microbial composition.

[00569] In some embodiments, the promoting agent may be a nutrient, for example, selected from sodium chloride, lithium chloride, sodium glycerophosphate, phenylethanol, mannitol, tryptone, and yeast extract. In some embodiments, the prebiotic is selected from the group consisting of short-chain fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid), glycerol, pectin-derived oligosaccharides from agricultural by-products, fructo-oligosaccharides (e.g., inulin-like prebiotics), galacto-oligosaccharides (e.g., raffinose), succinic acid, lactic acid, and mannan-oligosaccharides.

[00570] In some embodiments, the promoting agent may be a probiotic. The probiotic may be any known probiotic known in the art. Probiotics are live microorganisms that provide a health benefit to the host. In methods provided herein, probiotics may be applied topically to dermal and mucosal microbiomes, and/or probiotics may be orally administered to provide dermal and mucosal health benefits to the subject. Several strains of *Lactobacillus* have been shown to have systemic anti-inflammatory effects. Studies have shown that certain strains of *Lactobacillus reuteri* induce systemic anti-inflammatory cytokines, such as interleukin (IL)-10. Soluble factors from *Lactobacillus reuteri* inhibit production of pro-inflammatory cytokines. *Lactobacillus paracasei* strains have been shown to inhibit neutrogenic inflammation in a skin model Kober et al., 2015, Int J Women's Dermatol 1(2015) 85-89. In human dermal fibroblasts and hairless mice models, *Lactobacillus Plantarum* has been shown to inhibit UVB-induced matrix metalloproteinase 1 (MMP-1) expression to preserve procollagen expression in human fibroblasts. Oral administration of *L. plantarum* in hairless mice histologic samples demonstrated that *L. plantarum* inhibited MMP-13, MMP-2, and MMP-9 expression in dermal tissue.

[00571] Clinically, the topical application of probiotics has also been shown to modify the barrier function of the skin with a secondary increase in antimicrobial properties of the skin. *Streptococcus thermophiles* when applied topically has been shown to modify the barrier function of the skin with a secondary increase in antimicrobial properties of the skin. *Streptococcus thermophiles* when applied topically has been shown to increase ceramide production both in vitro and in vivo. Ceramides trap moisture in the skin, and certain ceramide sphingolipids, such as phytosphingosine (PS), exhibit direct antimicrobial activity against *P. acnes*. Kober et al., 2015, Int J Women's Dermatol 1(2015) 85-89.

[00572] Two clinical trials of topical preparations of probiotics have assessed their effect on acne. *Enterococcus fecalis* lotion applied to the face for 8 weeks resulted in a 50% reduction of inflammatory lesions was noted compared to placebo. A reduction in acne count, size, and associated erythema was noted during a clinical study of *Lactobacillus plantarum* topical extract. Kober et al., 2015, Int J Women's Dermatol 1(2015) 85-89.

[00573] Clinical trials of topical probiotics have evaluated their effect on mucosal systems. In one study, *Streptococcus salivarius* was administered by nasal spray for the prevention of acute otitis media (AOM). If the nasopharynx was successfully colonized, there was significant effect on reducing AOM. Marchisio et al. (2015). Eur. J. Clin. Microbiol. Infect. Dis. 34, 2377–2383. In another trial, sprayed application of *S. sanguinis* and *L. Rhamnosus* decreased middle ear fluid in children with secretory otitis media. Skovbjerg et al. (2008). Arch. Dis. Child. 94, 92–98.

[00574] The probiotic may be a topical probiotic or an oral probiotic. The probiotic may be, for example, a different genus and species than the undesirable microorganism, or of the same genus but different species, than the undesirable microorganism. The probiotic species may be a different genus and species than the target microorganism. The probiotic may or may not be modified to comprise a kill switch molecular modification. The probiotic may be selected from a *Lactobacillus* spp, *Bifidobacterium* spp. *Streptococcus* spp., or *Enterococcus* spp. The probiotic may be selected from *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*,

Lactobacillus casei, *Lactobacillus plantarum*, *Lactococcus lactis*, *Streptococcus thermophiles*, *Streptococcus salivarius*, or *Enterococcus faecalis*.

[00575] The promoting agent may include a protein stabilizing agent such as those disclosed in an incorporated by reference from U.S. Pat. No. 5,525,336 is included in the composition. Non-limiting examples include glycerol, trehalose, ethylenediaminetetraacetic acid, cysteine, a cyclodextrin such as an alpha-, beta-, or gamma-cyclodextrin, or a derivative thereof, such as a 2-hydroxypropyl beta-cyclodextrin, and proteinase inhibitors such as leupeptin, pepstatin, antipain, and cystatin.

[00576] The promoting agent may include a humectant. Non-limiting examples of humectants include glycerin, sorbitol, sodium 2-pyrrolidone-5-carboxylate, soluble collagen, and dibutylphthalate.

[00577] Compositions

[00578] Compositions are provided comprising a synthetic microorganism and a pharmaceutically acceptable carrier, diluent, emollient, binder, excipient, lubricant, sweetening agent, flavoring agent, buffer, thickener, wetting agent, or absorbent.

[00579] Pharmaceutically acceptable diluents or carriers for formulating the composition are selected from the group consisting of water, saline, phosphate buffered saline, or a solvent. The solvent may be selected from, for example, ethyl alcohol, toluene, isopropanol, n-butyl alcohol, castor oil, ethylene glycol monoethyl ether, diethylene glycol monobutyl ether, diethylene glycol monoethyl ether, dimethyl sulphoxide, dimethyl formamide and tetrahydrofuran. The carrier or diluent may further comprise one or more surfactants such as i) Anionic surfactants, such as metallic or alkanolamine salts of fatty acids for example sodium laurate and triethanolamine oleate; alkyl benzene sulphones, for example triethanolamine dodecyl benzene sulphonate; alkyl sulphates, for example sodium lauryl sulphate; alkyl ether sulphates, for example sodium lauryl ether sulphate (2 to 8 EO); sulphasuccinates, for example sodium dioctyl sulphosuccinate; monoglyceride sulphates, for example sodium glyceryl monostearate monosulphate; isothionates, for example sodium isothionate; methyl taurides, for example Igepon T; acylsarcosinates, for example sodium myristyl sarcosinate; acyl peptides, for example Maypons and lamepons; acyl lactylates, polyalkoxylated ether glycollates, for example trideceth-7 carboxylic acid; phosphates, for example sodium dilauryl phosphate; Cationic surfactants, such as amine salts, for example sapamin

hydrochloride; quaternary ammonium salts, for example Quaternium 5, Quaternium 31 and Quaternium 18; Amphoteric surfactants, such as imidazol compounds, for example Miranol; N-alkyl amino acids, such as sodium cocaminopropionate and asparagine derivatives; betaines, for example cocamidopropylebetaine; Nonionic surfactants, such as fatty acid alkanolamides, for example oleic ethanolamide; esters or polyalcohols, for example Span; polyglycerol esters, for example that esterified with fatty acids and one or several OH groups; Polyalkoxylated derivatives, for example polyoxy:polyoxyethylene stearate; ethers, for example polyoxyeth lauryl ether; ester ethers, for example Tween; amine oxides, for example coconut and dodecyl dimethyl amine oxides. In some embodiments, more than one surfactant or solvent is included.

[00580] The composition may include a buffer component to help stabilize the pH. In some embodiments, the pH is between 4.5-8.5. For example, the pH can be approximately 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8.0, including any value in between. In some embodiments, the pH is from 5.0 to 8.0, 6.0 to 7.5, 6.8 to 7.4, or about 7.0. Non-limiting examples of buffers can include ACES, acetate, ADA, ammonium hydroxide, AMP (2-amino-2-methyl-1-propanol), AMPD (2-amino-2-methyl-1,3-propanediol), AMPSO, BES, BICINE, bis-tris, BIS-TRIS propane, borate, CABS, cacodylate, CAPS, CAPSO, carbonate (pK1), carbonate (pK2), CHES, citrate (pK1), citrate (pK2), citrate (pK3), DIPSO, EPPS, HEPPS, ethanolamine, formate, glycine (pK1), glycine (pK2), glycylglycine (pK1), glycylglycine (pK2), HEPBS, HEPES, HEPPSO, histidine, hydrazine, imidazole, malate (pK1), malate (pK2), maleate (pK1), maleate (pK2), MES, methylamine, MOBS, MOPS, MOPSO, phosphate (pK1), phosphate (pK2), phosphate (pK3), piperazine (pK1), piperazine (pK2), piperidine, PIPES, POPSO, propionate, pyridine, pyrophosphate, succinate (pK1), succinate (pK2), TABS, TAPS, TAPSO, taurine (AES), TES, tricine, triethanolamine (TEA), and Trizma (tris). Excipients may include a lactose, mannitol, sorbitol, microcrystalline cellulose, sucrose, sodium citrate, dicalcium phosphate, phosphate buffer, or any other ingredient of the similar nature alone or in a suitable combination thereof.

[00581] The microbial composition may include a binder may, for example, a gum tragacanth, gum acacia, methyl cellulose, gelatin, polyvinyl pyrrolidone, starch or any other ingredient of the similar nature alone or in a suitable combination thereof; excipients selected from the group consisting of agar-agar, calcium carbonate, sodium carbonate, silicates, alginic acid, corn starch, potato tapioca starch, primogel or any other

ingredient of the similar nature alone or in a suitable combination thereof; lubricants selected from the group consisting of a magnesium stearate, calcium stearate, talc, solid polyethylene glycols, sodium lauryl sulfate or any other ingredient of the similar nature alone; glidants selected from the group consisting of colloidal silicon dioxide or any other ingredient of the similar nature alone or in a suitable combination thereof; a stabilizer selected from the group consisting of such as mannitol, sucrose, trehalose, glycine, arginine, dextran, or combinations thereof; an odorant agent or flavoring selected from the group consisting of peppermint, methyl salicylate, orange flavor, vanilla flavor, or any other pharmaceutically acceptable odorant or flavor alone or in a suitable combination thereof; wetting agents selected from the group consisting of acetyl alcohol, glyceryl monostearate or any other pharmaceutically acceptable wetting agent alone or in a suitable combination thereof; absorbents selected from the group consisting of kaolin, bentonite clay or any other pharmaceutically acceptable absorbents alone or in a suitable combination thereof; retarding agents selected from the group consisting of wax, paraffin, or any other pharmaceutically acceptable retarding agent alone or in a suitable combination thereof.

[00582] The microbial composition may comprise one or more emollients. Non-limiting examples of emollients include stearyl alcohol, glyceryl monoricinoleate, glyceryl mono stearate, propane-1,2-diol, butane-1,3-diol, mink oil, cetyl alcohol, isopropyl isostearate, stearic acid, isobutyl palmitate, isocetyl stearate, oleyl alcohol, isopropyl laurate, hexyl laurate, decyl oleate, octadecan-2-ol, isocetyl alcohol, cetyl palmitate, dimethylpolysiloxane, di-n-butyl sebacate, isopropyl myristate, isopropyl palmitate, isopropyl stearate, butyl stearate, polyethylene glycol, triethylene glycol, lanolin, sesame oil, coconut oil, arrachis oil, castor oil, acetylated lanolin alcohols, petroleum, mineral oil, butyl myristate, isostearic acid, palmitic acid, isopropyl linoleate, lauryl lactate, myristyl lactate, decyl oleate, myristyl myristate.

[00583] The microbial composition may include a thickener, for example, where the thickener may be selected from hydroxyethylcelluloses (e.g. Natrosol), starch, gums such as gum arabic, kaolin or other clays, hydrated aluminum silicate, fumed silica, carboxyvinyl polymer, sodium carboxymethyl cellulose or other cellulose derivatives, ethylene glycol monostearate and sodium alginates. The microbial composition may include preservatives, antiseptics, pigments or colorants, fragrances, masking agents, and carriers, such as water and lower alkyl, alcohols, such as those disclosed in an incorporated by reference from U.S. Pat. No. 5,525,336 are included in compositions.

[00584] The microbial compositions for topical administration may be provided in liquid, solution, suspension, cream, lotion, ointment, gel, or in a solid form such as a powder, tablet, or troche for suspension immediately prior to administration. The compositions for topical use may also be provided as hard capsules, or soft gelatin capsules, wherein the benign and/or synthetic microorganism is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil. Powders and granulates may be prepared using the ingredients mentioned above under tablets and capsules for dissolution in a conventional manner using, e.g., a mixer, a fluid bed apparatus, lyophilization or a spray drying equipment. A dried microbial composition may administered directly or may be for suspension in a carrier. When the composition is in a powder form, the powders may include chalk, talc, fullers earth, colloidal silicon dioxide, sodium polyacrylate, tetra alkyl and/or trialkyl aryl ammonium smectites and chemically modified magnesium aluminum silicate in a carrier. When the composition is in a powder form, the powders may include chalk, talc, fullers earth, colloidal silicon dioxide, sodium polyacrylate, tetra alkyl and/or trialkyl aryl ammonium smectites and chemically modified magnesium aluminum silicate

[00585] The microbial composition may exhibit a stable CFU losing less than 30%, 20%, 10% or 5% cfu over at least one, two, three months, six months, 12 months 18 months, or 24 months when stored at frozen, refrigerated or preferably at room temperature.

[00586] Kits

[00587] Any of the above-mentioned compositions or synthetic microorganisms may be provided in the form of a kit. In some embodiments, a kit comprises a container housing live bacteria or a container housing freeze-dried live bacteria. Kits can include a second container including media. Kits may also include one or more decolonizing agents. Kits can also include instructions for administering the composition. In certain embodiments, instructions are provided for mixing the bacterial strains with other components of the composition. In some embodiments, a kit further includes an applicator to apply the microbial composition to a subject.

[00588] Dose

[00589] In certain embodiments, a composition is provided for topical administration that is a solution composition, or for reconstitution to a solution

composition. In one embodiment, composition may include from about 1×10^5 to 1×10^{12} cfu/ml, 1×10^6 to 1×10^{10} cfu/ml, or 1.2×10^7 to 1.2×10^9 CFU/mL of the synthetic microorganism in an aqueous solution, such as phosphate buffered saline (PBS). Lower doses may be employed for preliminary irritation studies in a subject.

[00590] Preferably, the subject does not exhibit recurrence of the undesirable microorganism as evidenced by swabbing the subject at the at least one site after at least 2, 3, 4, 6, 10, 15, 22, 26, 30 or 52 weeks after performing the initial administering step.

[00591] Nanofactory

[00592] In some embodiments, methods are provided to create production of a desired substance at the site of the microbiome (nanofactory). Synthetic microorganisms are provided that may comprise a nanofactory molecular modification. The term “nanofactory” refers to a molecular modification of a target microorganism that results in the production of a product - either a primary product such as a protein, enzyme, polypeptide, amino acid or nucleic acid, or a secondary product such as a small molecule to produce a beneficial effect. The product may be secreted from the synthetic microorganism or may be in the form of an inclusion body. Such nanofactory bacterial strains have the potential to provide to the host subject a wide range of durable benefits including: (i) the acquisition of cellular products and enzymes for which the host was previously deficient and; (ii) the acquisition of a delivery system of a microbially manufactured small molecule, polypeptide or protein pharmaceuticals for diverse therapeutic and prophylactic benefit. Such nanofactory bacterial strains when durably integrated into the biome as described herein would provide a useful durable alternative steady state production of product than direct product application.

[00593] Methods and synthetic microorganisms are provided herein to replace existing colonization by an undesirable microorganism with a synthetic bacterial strain comprising a nanofactory molecular modification for the production or consumption of a primary or secondary product, where the target microorganism may be a strain of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus*

viridans, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* and *Lactobacillus iners*.

[00594] The nanofactory molecular modification in a synthetic microorganism may be used to assist its host subject, i.e., a patient with a deficit of some primary (anabolic or catabolic) or secondary metabolic pathway or any other ailment stemming from the over or under abundance of some small molecule or macromolecule such as an enzyme. The nanofactory molecular modification may encode an enzyme, amino acid, metabolic intermediate, or small molecule. The nanofactory molecular modification may confer a new production (synthesis) or metabolic function into the host microbiome, such as the ability to endogenously synthesize or metabolize specific compounds, or synthesize enzymes or other active molecules to operate within the exogenous microbiome.

[00595] The microorganism will carry a nanofactory selected from a biosynthetic gene, biosynthetic gene cluster, or gene(s) coding for one or multiple enzymes under the control of a differentially regulated, inducible or constitutively regulated promoter. The synthetic microorganism comprising a nanofactory is to be administered to at the at least one site of the body be it dermal, mucosal, or other site as a singular agent or in conjunction with a second, third or fourth synthetic microorganism that help the first synthetic microorganism restore the loss of function on or in the host subject.

[00596] In one example, a synthetic microorganism comprising a nanofactory may be used for restoration of function by the production of intercellularly active factors, for example, microbial supplementation of digestive enzymes in patients with exocrine pancreatic insufficiency by secreted recombinant enzymes in the small intestine. The pancreas is a vital organ and plays a key role in digestion. Exocrine pancreatic insufficiency (EPI) is caused by prolonged damage to the pancreas, which leads to the reduction or absence of quintessential digestive enzymes in the small intestine that primarily breakdown fats and carbohydrates. The loss of these enzymes can lead to a wide breadth of symptoms and depends on the severity of the EPI. The small intestine's pH level in the proximal small intestine (duodenum) is lower than that of the distal region. This shift in environment leads to microbial niche occupation that is pH

dependent. This pH dependency has naturally selected for duodenum commensal bacteria that could be molecularly modified to become synthetic microorganisms, which would intrinsically localize themselves to that region of the gastrointestinal tract. The stomach and upper two-thirds of the small intestine contain acid tolerant Lactobacilli and Streptococci (Hao, Wl, Lee YK. Microflora of the gastrointestinal tract: a review. Methods Mol. Biol. 2004, **268**, 491-502) and could be isolated from healthy donors. By knocking in recombinant lipases, amylases and/or proteases with secretory signaling sequences, the colonization of the duodenum by the synthetic microorganisms could restore digestive function in patients suffering from EPI.

[00597] In another example of a nanofactory, a synthetic microorganism comprising a nanofactory may be used for restoration of function by the production of intracellularly active factors. For example, protecting a subject suffering from phenylketonuria (PKU) by eliminating phenylalanine in the gastrointestinal tract. Phenylalanine is an essential amino acid, meaning that the human body cannot produce it and must acquire it through nourishment. Once in the body, the breakdown of phenylalanine is carried out by one protein, phenylalanine hydroxylase (PAH). The inheritable genetic disorder known as phenylketonuria (PKU) is caused by mutations in the gene coding for PAH, which results in the build up of phenylalanine in the body. One of the most common approaches to circumvent this accumulation is to avoid phenylalanine rich foods. Alternatively, a synthetic microorganism that has been molecularly modified to breakdown phenylalanine intracellularly can be introduced into the gastrointestinal tract. This synthetic microorganism constitutes a PAH nanofactory, breaking down phenylalanine before it has a chance to enter the body of the host with PKU.

[00598] In another example of a nanofactory, a synthetic bacteria may be derived from a target commensal bacteria from the skin microbiota may comprising a nanofactory molecular modification. The target commensal skin or mucosal bacterium may be, e.g., a *Staphylococcus spp.*, *Streptococcus spp.*, or a *Cutibacterium spp.* For example, *Staphylococcus epidermidis* may be the target microorganism because it is found in multiple dermal or mucosal environmental types. Engineering a synthetic *S. epidermidis*, given its ability to persist in different environments, would allow for the development and optimization of multiple kinds of delivery techniques and locations.

[00599] In one example, a synthetic *S. epidermidis* strain may comprise a nanofactory molecular modification to produce testosterone for men suffering from male hypogonadism. The production of testosterone could be accomplished by: (i) introduction of the entire sterol biosynthetic pathway with the additional enzymes necessary to generate testosterone, or (ii) introduction of the partial sterol biosynthetic pathway and having the necessary precursor molecules in the carrying medium, i.e., farnysel, squalene, cholesterol etc, so that testosterone could be assembled in the synthetic bacterium. In another example, a synthetic *S. epidermidis* strain could comprise a nanofactory molecular modification for production of nicotine; this synthetic strain could be applied as a transdermal therapy to help with smoking cessation. This synthetic strain may include a molecular modification to include one or more biosynthetic pathways found in the Solanaceae family of plants, and optionally further include a molecular modification for the enhancement of intrinsic pathways of precursor molecules, i.e., aspartic acid, ornithine etc.

[00600] In a further example of a nanofactory, a synthetic *S. epidermidis* strain may comprise a nanofactory molecular modification for the production of scopolamine. Scopolamine is currently delivered via an extended release transdermal patch for treatment of motion sickness and postoperative prophylaxis. This strain would need to carry the biosynthetic pathways found in the Solanaceae family of plants and possibly the enhancement of intrinsic pathways of precursor molecules.

[00601] As another example, a synthetic *S. epidermidis* strain may comprise a nanofactory molecular modification for the production of capsaicin to alleviate pain stemming from post-herpetic neuralgia, psoriasis or other skin related disorders.

[00602] In another example, the target microorganism is a *Streptococcus mutans* strain, which may have one or more of a kill switch, V-block, or nanofactory molecular modification. Dental caries and dental plaque are among the most common diseases worldwide, and are caused by a mixture of microorganisms and food debris. Specific types of acid-producing bacteria, especially *Streptococcus mutans*, colonize the dental surface and cause damage to the hard tooth structure in the presence of fermentable carbohydrates e.g., sucrose and fructose. Dental caries and dental plaque are among the most common diseases worldwide, and are caused by a mixture of microorganisms and food debris. Specific types of acid-producing bacteria, especially *Streptococcus mutans*, colonize the dental surface and cause damage to the hard tooth structure in the presence

of fermentable carbohydrates e.g., sucrose and fructose. Forrsten et al, *Nutrients*, 2010 Mar; 2(3):290-298. In some embodiments, the target microorganism is *S. mutans* having a KS and/or a nanofactory knock out for reducing acid production in presence of sucrose, fructose, or other fermentable carbohydrates.

[00603] Further examples of nanofactory molecular modifications in a synthetic microorganism to address dermatological and cosmetic uses include: (i) hyaluronic acid production in *Staphylococcus epidermidis* for atopic dermatitis or dry skin, (ii) alpha-hydroxy acid production in *Staphylococcus epidermidis* to reduce fine lines and wrinkles as well as lessen irregular pigmentation, (iii) salicylic acid production in *Cutibacterium acnes* to reduce acne, (iv) arbutin production in *Staphylococcus epidermidis* (arbutin and its metabolite hydroquinone function as skin lightening agents by melanin suppression, (v) Kojic acid (produced by several fungi including *Aspergillus oryzae*) in *Staphylococcus epidermidis* to lighten skin pigmentation, (vi) Retinoid production by *Staphylococcus epidermidis* for the reduction of fine lines and wrinkles, (vii) L-ascorbic acid (Vitamin C) production in *Staphylococcus epidermidis* for the stimulation of collagen and antioxidant effects on the skin, (viii) copper peptide (GHK-Cu) production in *Staphylococcus epidermidis* for stimulation of collagen and elastin production and reduction of scar formation, (ix) alpha lipoic acid production in *Staphylococcus epidermidis* for beneficial antioxidant effects on the skin., and (x) dimethylaminoethanol production in *Staphylococcus epidermidis* for reducing fine lines and wrinkles.

[00604] *Cutibacterium acnes* is a dominant bacteria living on the skin, and has been associated with both healthy skin and various diseases. This is another organism and niche available for enhancing and strengthening with modern molecular biology techniques. Studies have shown that the levels of *C. acnes* are similar between healthy skin and skin laden with acne. Dréno, B., et al. "Cutibacterium acnes (Propionibacterium acnes) and acne vulgaris: a brief look at the latest updates." *Journal of the European Academy of Dermatology and Venereology* 32 (2018): 5-14. This indicates that just lowering the number of viable *C. acnes* on a person's skin will not help to alleviate the disease or symptoms. Instead, other strains of *C. acnes* or other members of the dermal and subcutaneous microbiome can be altered to mitigate the mechanisms that certain *C. acnes* strains use to cause disease. The isolates that showed to have the greatest association with increased acne severity also have been shown to produce higher quantities of propionic and butyric acid. Beylot, C., et al. "Propionibacterium acnes: an

update on its role in the pathogenesis of acne." *Journal of the European Academy of Dermatology and Venereology* 28.3 (2014): 271-278.

[00605] Another example of a nanofactory molecular modification includes another strain of *C. acnes* that is modified to have an increased appetite for short chain fatty acids, such as propionic and butyric acid, thereby removing the inflammatory chemical secretions from the virulent strain rendering it less toxic. The carbon rich fatty acids could be used to induce a heterologous pathway and used as precursors for vitamin synthesis or other organic compounds beneficial for the skin or microbiome that inhabits that location.

[00606] In another example, in *S. epidermidis* lipoteichoic acid has shown to help mitigate the inflammatory response of *Propionibacterium acnes* (i.e., *Cutibacterium acnes*) by inducing miR-143. Xia, Xiaoli, et al. "Staphylococcal LTA-induced miR-143 inhibits *Propionibacterium acnes*-mediated inflammatory response in skin." *Journal of Investigative Dermatology* 136.3 (2016): 621-630. A synthetic microorganism comprising a nanofactory molecular modification producing lipoteichoic acid which inhibits *C. acnes*-induced inflammation via induction of miR-143 may be employed. The nanofactory may be used to modulate inflammatory responses by *S. epidermidis* at the site of acne vulgaris for management of *C. acnes*-induced inflammation. This pathway is just one example of a useful product that could be made from short chain fatty acids that when left alone cause inflammation and skin irritation.

[00607] In another example, inflammation and an increase in temperature are factors involved in the disease caused by *C. acnes*, they could be used as signals to induce previously silent heterologous pathways in an engineered strain. A temperature increase (signalling a sealed pore and progressing localized disease state) could induce in the virulent strain or another commensal microbe, the transcription and translation of a non-immune stimulating lipase (or other enzyme) that is capable of degrading the sebum to the point of reopening a clogged pore allowing the location to resume its normal growth conditions.

[00608] In a further example, a synthetic *Lactobacillus* spp. such as *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* or *Lactobacillus iners* – which are common dominant species present in the female vaginal vault may be engineered to

comprises a nanofactory molecular modification that produces estradiol in the vaginal vault of post-menopausal women.

[00609] Methods and synthetic microorganisms are provided herein to replace existing colonization by an undesirable microorganism with a synthetic bacterial strain comprising a nanofactory molecular modification for the production or consumption of a primary or secondary product, for example, selected from an enzyme, nicotine, aspartic acid, ornithine, propionic acid, butyric acid, hyaluronic acid, an alpha-hydroxy acid, L-ascorbic acid, a copper peptide, alpha-lipoic acid, salicylic acid, arbutin, Kojic acid, scopolamine, capsaicin, a retinoid, dimethylaminoethanol, lipoteichoic acid, testosterone, estradiol, and progesterone.

[00610] The durable integration of a synthetic bacterial strain that is able to produce by means of a nanofactory molecular modification or synthetic addition to its genome, a substance, material, or product, or products, that are beneficial to the host at the site of the microbiome integration or at distant sites in the host following absorption may be tailored to the desired indication. Depending upon whether the synthetic nucleotide change is incorporated directly into the bacterial genome, or whether it was introduced into plasmids, the duration of the effect of the nanofactory production could range from short term (with non-replicating plasmids for the bacterial species,) to medium term (with replicating plasmids without addiction dependency) to long term (with direct bacterial genomic manipulation).

[00611] Virulence block

[00612] In some embodiments, methods are provided to replace existing colonization with a synthetic bacterial strain which cannot accept genetic transfer of undesired virulence or antibiotic resistance genes. Synthetic microorganisms are provided that may comprise a “virulence block” or “V-block”. The term “virulence block”, or “V-block” refers to a molecular modification of a microorganism that results in the organism have decreased ability to accept foreign DNA from other strains or species effectively resulting in the organism having decreased ability to acquire exogenous virulence or antibiotic resistance genes.

[00613] Methods are provided herein to replace existing colonization by an undesirable microorganism with a synthetic bacterial strain comprising a V-block molecular modification which cannot accept genetic transfer of undesired virulence or

antibiotic resistance genes, where the target microorganism may be a strain of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*.

[00614] One of the major concerns with regard to infectious diseases is commonly called “horizontal gene transfer” with potential bacterial pathogens acquiring either exogenous virulence protein genes or antimicrobial resistance genes. The acquisition may result from transfer of these genes from other bacteria strains or species in the local microbiome environment. As it is common for invasive bacterial pathogens to initially be a part of the colonizing bacterial microbiome on skin or mucosal surfaces prior to causing disease, it would be of great practical benefit to be able to imbue these colonizing strains with the inability to accept foreign bacterial DNA into the bacterial genome. The process to accomplish this in a durably integrated synthetic bacterial strain has been termed called “virulence block.” Such a “virulence block” manipulated strain would be able to be integrated into the microbiome after a decolonization event and then through the process of competitive exclusion, remain for a time as the dominant strain within that particular niche without reacquiring undesired virulence or antibiotic resistance characteristics. Such a concept carried out on potential pathogens within the microbiome would result in a stable microbiome which could acquire neither virulence nor antimicrobial resistance genes in the horizontal transfer manner, rendering the totality of the microbiome more robust and with lowered conversion potential.

[00615] The V-block is a molecular modification that may be employed in a synthetic microorganism in order to suppress virulence or horizontal gene transfer from an undesirable microorganism. The V-block molecular modification may be created in a target microorganism by: (i) gene knockout (excise or remove) of one or more known

virulence genes, (ii) frameshift of a virulence region (adding or subtracting base pairs to 'break' the coding frame), (iii) exogenous silencing of virulence regions using inducible promoter or constitutive promoter (embedded in the DNA genome, but functions in RNA) – like antitoxin strategy, production of CRISPR-CAS9 or other editing proteins to digest incoming virulence genes using guide RNA which may be linked to an inducible promoter or constitutive promoter, or (iv) by a restriction modification (RM) such as a methylation system to turn the organism's 'innate immune system' to recognize and destroy incoming virulence genes by class of molecule. Any of these methods may be employed in order to increase resistance to horizontal gene transfer. Gene editing methods for constructing a V-block may include NgAgo, mini-Cas9, CRISPR-Cpf1, CRISPR-C2c2, Target-AID, Lambda Red, Integrases, Recombinases, or use of Phage. The virulence block may be operably linked to a constitutive promoter in the synthetic microorganism. The virulence block molecular modification may prevent horizontal gene transfer of genetic material from a virulent microorganism.

[00616] The gene cassette conferring antibiotic resistance to strains of *Staphylococcus aureus* (SA) may be integrated into the recipient cell's genome at a particular site. This site could be deleted or changed in a cell's genome, making the landing site no longer available for the incoming DNA sequence. This has been shown not to interfere with SA's ability to grow, and would make the acquisition of the resistance cassette by the organism much less likely to occur

[00617] The V-block molecular modifications may cause the removal or neutralization of virulence factors, resistance loci or cassettes, toxins or toxigenic functions, or other undesired attributes of the biologically integrated microorganism.

[00618] A virulence block in the form of Cas9 recognition system for sequences consistent with known virulence factors or antibiotic resistance genes in *Staphylococcus aureus* may be used to protect strains of *Staphylococcus aureus* Live Biotherapeutic Products from acquiring additional virulence factors and resistances to antibiotic classes, thus rendering them as safe as initially approved and manufactured.

[00619] CRISPR is a native adaptive immune system for prokaryotic cells that has evolved over time to help defend against phage attacks. The system uses short DNA sequences complementary to phage DNA (or any target DNA) sequences to target

incoming DNA and digest the strand before it can be incorporated into the genome of the living cell.

This same technology may be engineered to target DNA sequences that are non-threatening to the bacterial cell, but once acquired allow the organism to cause disease and persist in environments that were previously less habitable. Through integrating the Cas-9 enzyme into the genome, or harnessing the endogenous Cas-9 if available, it is possible to introduce into the genome constitutively expressed guide RNAs that target antibiotic resistance genes. If the targeted sequences are ever introduced to the cell through horizontal gene transfer or otherwise, the incoming DNA will be cut up and unable to integrate into the genome or produce a functional peptide. If the genes become integrated into the genome before the CRISPR-Cas system can target it, the engineered CRISPR-Cas system will find it in the genome and cut the sequences at the targeted location, thus producing a non-viable cell and stopping the spread of antibiotic resistance cassettes.

[00620] The CRISPR system can also be used to target RNA sequences with the result of silencing gene expression. Instead of recognition sequences targeting the DNA sequence of antibiotic resistance or virulence genes, the recognition sequences can be designed to target mRNA. If Cas9 and the targeting guide RNAs are constitutively expressed in a cell that receives the abxR or virulence genes, the translation will be interrupted by the engineered CRISPR system impeding protein formation and the ability of the cell to use the targeted genes.

[00621] Yet another method of gene silencing in prokaryotes that may be used to target the expression of virulence or antibiotic resistant genes is to design and constitutively express regulatory RNAs that target the mRNA transcript, usually at the RBS. These would be integrated into and constitutively expressed from the genome to create a synthetic organism. The regulatory RNA is a short sequence (>100bp) and is complementary to the 5' untranslated region (UTR) of the mRNA transcript of the abxR or virulence gene. The constitutive expression of the short sequences should not be metabolically taxing for the organism, and will have the result of blocking translation of the targeted mRNA into a protein. The engineered RNA will sufficiently block the cells ability to utilize the targeted antibiotic resistance gene if and when it is received through horizontal gene transfer.

[00622] DNA methylation plays many important roles in prokaryotes and eukaryotes. One feature of DNA methylation allows a cell to distinguish its own DNA from foreign DNA. This makes editing and studying many wild type strains very difficult, because the organism's methylase systems recognize transformed plasmid DNA as foreign, and chew it up before it can be transcribed or integrated. Horizontal gene transfer can occur between organisms that have very similar methylation patterns because the incoming DNA looks very similar to the recipient's own DNA and it is not digested. Since the mechanism and genes responsible for adding methyl groups to specific sequences, and those that look for and cut improperly methylated DNA are known in a variety of bacterial strains, it is possible to create a synthetic organism that is capable of having a unique methylation pattern. This would serve to make all incoming DNA appear foreign to the synthetic organism and get digested before the organism can acquire the new traits. This would serve to render the horizontal gene transfer of virulence or antibiotic resistance genes into our synthetic organism a non-issue.

[00623] A V-Block in the form of a molecular disruption of one or more bacterial genomic cassette insertion sites in the synthetic microorganism can render the synthetic microorganism unable to acquire antibiotic class resistance genes from resident bacteria species that are cohabitating the biome. Such manipulation will also prevent the acquisition of virulence genes that could increase the possibility of invasive events across the bowel wall. The gene cassette conferring antibiotic resistance to strains of *Staph aureus* (SA) may be integrated into the recipient cell's genome at a particular site. This site could be deleted or changed in a cell's genome, making the landing site no longer available for the incoming DNA sequence. So long as the V-block is shown not to interfere with the synthetic microorganism's ability to grow, and would make the acquisition of the resistance cassette by the organism much less likely to occur.

[00624] EXAMPLES

[00625] **Example 1. Field Studies - Exclusionary Niche using Benign Microorganism:**

[00626] **Clinical Studies-Suppress and Replace**

[00627] A clinical study was designed to identify MRSA positive subjects, suppress the MRSA strain, replace the MRSA by administering BioplX-01 (i.e., MSSA

502a), and periodically retesting subjects for recurrence of MRSA. The study population was largely drawn from Meerut area Medical Personnel and Medical Students. No symptomatic subjects were enrolled in the study.

[00628] This is a “proof of principle” study, being performed with largely unimproved materials and methods – any result greater than 55% non-recurrence will be considered an indication of the potential efficacy of these methods. Any result at 80% or greater non-recurrence would be considered a strong indication of the current technical strength of this approach.

[00629] Study purpose and primary endpoints:

- 1) To determine the rate of asymptomatic *Staphylococcus aureus* and MRSA occurrence in the general population – Meerut, UP (North India) – and to qualify participants for further phases of this study;
- 2) Determine the rate of MRSA recurrence in BioPlx decolonized participants;
- 3) Determine the rate of MRSA recurrence in BioPlx01-WT recolonized participants;
- 4) Determine the durability of BioPlx01-WT in preventing MRSA recurrence (to 8 & 12 wks);
- 5) Acceptable study recurrence level = 40%, Target recurrence level = 20%.

[00630] The study results are evaluated against the published recurrence rates from peer-reviewed sources, averaging 45% recurrence, 55% non-recurrence.

[00631] Identification and solicitation of potential participants was performed with total participants enrolled and tested: n=765. Patients were drawn from the Medical Staff and Medical Students of: Meerut University Medical College – LLRM Medical College (MUMC Hospital), Harish Chandra Hospital, Murti Hospital, Silver Cross Hospital, JP Hospital, and Lokpriya Hospital, Dhanvantri Hospital, Jaswantrai Hospital. A paper disclosure, informed consent, and sign up document signed by all participants.

[00632] All 765 potential participants were swabbed (Nasal) by lab personnel. All swabs were plated onto a *Staphylococcus aureus* and a MRSA chromagar plate by lab personnel. All plates were incubated for 24 hours at 37°C, read and scored by the study supervisor personally. Photographs were taken of all plates at reading and labeled results.

[00633] The total *Staphylococcus aureus* nasal swab positive (MSSA and MRSA) participants was 162 or 21.18%, at the low end of expected rate for nasal swab only. The number of MSSA only (non-MRSA) participants was 97 or 12.68 %.

[00634] The number of MRSA positive participants was 65 or 8.50% of total tested population.

[00635] The MRSA positive participants (n = 65) were selected for the Efficacy Study by the study supervisor. The *Staphylococcus aureus* positive participants were selected for the irritation study by the study supervisor.

[00636] Efficacy Study was performed using BioPlx01-WT (10^8) in PBS.

[00637] Confirmed MRSA positive participants (n = 65) were advised as to the 12 week duration and commitment to the process. Study duration was extended to 6 months. Subjects for the Efficacy Study were divided as shown in Table 10.

[00638] Table 10. Efficacy Study

MRSA Positives Identified	n = 65
MRSA positive used in treatment groups - Decol/Recol	n = 34
MRSA positive used in negative controls - Decol only	n = 15
MRSA lost from study (Antibiotic use/drop-out)	n = 04
MRSA positive not used	n = 12

[00639] **Decolonization/Recolonization Process**

[00640] **Decolonization.**

[00641] A complete decolonization is performed on participants first. Following is confirmation of MRSA eradication in key sites. The total body decolonization is done with chlorhexidine, nasal decolonization is done with mupirocin, and gargling with Listerine original antiseptic as per the “Decolonization Protocol” section. After complete course of decolonization procedure (five days), a confirmation MRSA test will be administered to verify that no MRSA is present in key areas, and an *Staphylococcus aureus* test will be administered to gather information about post-colonization *Staphylococcus aureus* levels. Participants underwent five-day decolonization process, which was administered and observed by study personnel. Dermal decolonization was performed by study personnel and included (1) full body spray application of

chlorhexadine (4%), (2) nasal (mucosal) decolonization with mupriocine (2%), and (3) throat (mucosal) decolonization by application of Listerine, each once per day over 5 days. Participants undergo five-day decolonization process, administered and observed by BioPlx Pvt Ltd personnel.

Dermal - Chlorhexadine

Nasal (Mucosal) - Mupriocine

Throat (Mucosal) - Listerine

[00642] The participants undergo one full-body chlorhexidine bath that fully decolonizes the skin and hair. It is also true that chlorhexidine has a residual antibiotic activity that lasts as long as the outer layer of skin is present. A five-day waiting period ensures that the outer layer of skin has sloughed off and that when the subject is recolonized, BioPlx-01 is not being killed in the process.

[00643] Nasal Decolonization. To decolonize the nose and throat, the participants must use a five-day course of mupiricin antibiotics. This fully decolonizes the nares (nose).

[00644] Throat Decolonization. To decolonize the throat, the participants must gargle for 30 seconds every day with Original Listerine. This fully decolonizes the throat.

[00645] Successful decolonization is characterized by a negative MRSA result for nose, throat, and axilla (armpit). With successful decolonization only nasal follow-up testing is required at downstream timepoints. MRSA positive in nose or throat require second full round of decolonization procedure. Patients in this category do not proceed to next phase of study until decolonized. MRSA positive in axilla does not require second full round of decolonization and may proceed to next phase of study. Axilla site must now be included in all downstream MRSA testing.

[00646] Post-Decolonization Qualification Test N-T-H-A – *Staphylococcus aureus* and MRSA for each study Group (1,2,3). Swabs taken by Garg lab personnel. All swabs were plated onto a *Staphylococcus aureus* and a MRSA chromagar plate by Gard lab personnel. All plates were incubated in Dr. Garg's lab for 24 hours. All plates were read and scored by Dr. Garg personally. Photographs were taken of all plates at reading and labeled with Dr. Garg results. All data were recorded by BioPlx Pvt Ltd in paper and

digital form. All digital data are transmitted to BioPlx, Inc. for filing and entry into the records system. This procedure was used for all steps in Efficacy Study.

[00647] Recolonization was performed with application of 1.2×10^8 cfu/mL BioplX-01 in phosphate buffered saline (PBS), as described below, about 15 mL once per day for two consecutive days per the following schedule:

[00648] 1.2×10^8 RECOLONIZATION AND QC TESTING was performed two days back-to-back;

[00649] POST 1.2×10^8 RECOLONIZATION TESTING – one day;

[00650] POST 1.2×10^8 RECOLONIZATION TESTING – one week; and

[00651] Weekly Observation – week 2 and thereafter.

[00652] Post-Decolonization Qualification Test N-T-H-A – *Staphylococcus aureus* and MRSA was performed for each study Group (1, 2, 3).

[00653] Weekly observations included swabs of the subjects were taken by lab personnel. Anatomical sites sampled included nares, throat, axilla, hand.

[00654] All swabs were plated onto a *Staphylococcus aureus* and a MRSA chromagar plate by lab personnel. All plates were incubated for 24 hours at 37°C. All plates were read and scored by the study director personally. Photographs were taken of all plates at reading and labeled with results.

[00655] Negative controls. Post decolonization negative controls n = 15; ID#s: 0021, 0022, 0060, 0512, 0704, 0724, 0731, 0218, 0234, 0239, 0249, 0302, 0327, 0037, 0221. Post decolonization MRSA recurrence n = 15: Initial negative control run (sheet week 4 - Post-Decolonization average week 6) included MRSA positive n = 08; MRSA negative n = 07, resulting in Recurrence = 53%. A Final Negative Control run (sheet week 12 - Post-Decolonization average week 16) resulted in MRSA positive n = 09; and MRSA negative n = 06, with a recurrence = 60%.

[00656] Treatment Groups 1, 2, 3. Decolonized / Recolonized (8×10^8 cell concentration): 34. The Decolonized/Recolonized was divided into three groups for the study: GROUP 1 BioPlX01-WT (10^8) in PBS n = 10; ID#s: 0015, 0086, 0146, 0147, 0149, 0155, 0178, 0625, 0657, 0667. GROUP 2 BioPlX01-WT (10^8) in PBS n = 10; ID#s: 0063, 0075, 0124, 0138, 0172, 0325, 0444, 0478, 0483, 0538; and GROUP 3 BioPlX01-WT (10^8) in PBS n = 14 ID#s: 0064, 0112, 0158, 0232, 0336, 0488, 0497, 0498, 0499, 0552, 0574, 0692, 0725, 0735.

Post Decolonization/Recolonization MRSA Recurrence: 0; GROUP 1 = 0; GROUP 2 = 0; GROUP 3 = 0. Duration of post decolonization MRSA negative: 18 weeks = 16 cases: 0 recurrence; and 17 weeks = 18 cases: 0 recurrence.

[00657] Detectable Recolonization Performance

[00658] Subjects in the efficacy study were tested for *Staphylococcus aureus* positive results to detect presence of replacement BioPlx 01 WT using penicillinase disks. Results are shown in Table 11.

[00659] Table 11. *Staphylococcus aureus* Positives (NvTvHvA)

SA positives	Day/Week Post Colonization; +/total
97.1 % (Group 1 & 2 & 3)	01 day; 33/34
91.2 % (Group 1 & 2 & 3)	01 week; 31/34
100 % (Group 1 & 2 & 3)	02 week; 34/34
97.1 % (Group 1 & 2 & 3)	03 week; 33/34
91.2 % (Group 1 & 2 & 3)	04 week; 31/34
100 % (Group 1 & 2 & 3)	05 week; 34/34
88.2 % (Group 1 & 2 & 3)	06 week; 30/34
79.5 % (Group 1 & 2 & 3)	08 week; 27/34
67.7 % (Group 1 & 2 & 3)	10 week; 23/34
85.3 % (Group 1 & 2 & 3)	12 week; 29/34
100 % (Group 1 & 2 & 3)	14 week; 20/20

[00660] The study duration was extended to six months. At the conclusion of the study, *Staphylococcus aureus* positives were 100% showing a greater than 26 week total exclusionary effect of the BioPlx-01 MRSA decolonization/recolonization process with the BioPlx product as opposed to prior literature demonstrating 45% recurrence of *Staphylococcus aureus* nasal colonization at 4 weeks and 60% at 12 weeks with the standard decolonization method alone.

[00661] Irritation Studies

[00662] As described above, MRSA positive participants were selected for the Efficacy Study by the study supervisor (Dr. Garg). *Staphylococcus aureus* positive participants were selected for the Irritation Study by the study supervisor. MRSA patients require a lot of effort to screen for, so an attempt was made to preserve them for the main

efficacy evaluation of the study. Non-MRSA positive colonization rates are about 33%-66% of all screened participants, so there was a more plentiful supply of them. Because MRSA is an antibiotic resistant strain of *Staphylococcus aureus*, testing for irritation in *Staphylococcus aureus* positive participants is equivalent to testing for irritation in MRSA positive participants.

[00663] Irritation studies were performed on 55 *Staphylococcus aureus* positive subjects by topically administering about 5 mL of BioPlx-01 (502a), at 1.2×10^7 CFU/mL in PBS, to the right forearm. The left arm served as a negative control. Forearms were observed and photographed by study personnel at day 1, day 4 and day 7 post-application for redness or pustule development. No suppression step was performed during the irritation study. No irritation or adverse events were observed.

[00664] Culture conditions

[00665] The efficacy studies used BioPlx-01 (1.2×10^8 CFU/mL) in PBS (Fisher) BP2944100 phosphate buffered saline tablets dissolved in water to provide 100 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4 at 25°C.

[00666] Master stocks were prepared as follows. BioPlx-01 strain was streaked onto tryptic soy agar (TSA) plates in quad streak fashion. After 20 h at 37°C, a fresh bolus of cells was used to aseptically inoculate a flask of sterile tryptic soy broth (TSB). This culture was incubated at 37°C with agitation at 250 rpm for 18 h. Sterile 50% glycerol was added to the culture to 5% (v/v) final and the batch was aliquotted into sterile 50 mL polypropylene screwcap tubes. The aliquots were frozen at -20°C. For quality control, one aliquot was thawed, fully resuspended by vigorous shake-mixing, and diluted for the determination of colony forming units (CFU) per mL by incubation on Brain Heart Infusion (BHI) agar plates for 18 h at 37°C. CFU values were calculated from dilution-corrected colony counts. A batch of the concentrated BioPlx-01 master stock produced in this way contained 8×10^9 CFU/mL of BioPlx-01. The phenotypic identity of the strain was confirmed by incubation on HiChrom staphylococcal chromogenic indicator medium for 18 h at 37°C, which produced only the expected green colonies. The material did not produce colonies when incubated on MRSA chromogenic indicator plates.

[00667] Preparation of working stock for the efficacy study 1.2×10^8 CFU/mL

[00668] One 10 mL aliquot of concentrated BioPlx-01 stock that is at 8×10^9 CFU/mL was completely thawed and then shaken for a full 1 minute to mix. 8.5 mL of this solution were added to 275 mL of sterile (room temperature) PBS, generating a 2.4×10^8 CFU/mL stock. This was mixed well by inversion and stored at 4°C until use. As used in the efficacy studies, to provide PBS matrix 1.2×10^8 working solution-BioPlx-01, a vial of the " 2.4×10^8 CFU/mL" solution was mixed by vigorous inversion and 200 mL of it was added to 200 mL PBS to create a " 1.2×10^8 CFU/mL working solution-BioPlx-01". This latter solution was the material applied to subjects in efficacy studies. The bottle was tightly capped, mixed by shaking, and stored at 4C until use.

[00669] Example 2. Selection of one or more inducible promoters

In this example, promoter candidates were evaluated. The fold-induction and basal expression of 6 promoter candidates in a MSSA strain BioPlx-01 were evaluated by incubation with human whole blood and serum. Expression was normalized to a housekeeping gene (*gyrB*) and was compared with that in cells growing logarithmically in liquid tryptic soy broth (TSB) media.

[00670] The BioPlx-01 was grown to mid log phase (2 OD/mL) and then washed in large volume and shifted to freshly collected serum and heparinized blood from donor TK.

[00671] The samples were incubated in slowly agitating vented flask at 125 rpm; and samples were removed for RNA isolation at 15, 45, or 75 min at 37°C. The collected bacteria were washed, and RNA was extracted using Qiagen Allprep kit, eluted and the RNA frozen. Coding DNA (cDNA) was prepared from RNA and target gene expression evaluated by real time PCR (Taqman) in an ABI 7500 Fast instrument.

[00672] Relative RNA levels were determined by interpolation against a standard curve run on a common cDNA sample that was serially diluted and tested with primer/probes specific for ORFs driven by each of 5 putative serum-responsive promoters (*P_{hlgA}*, *P_{leuA}*, *P_{sstA}*, *P_{sirA}*, *P_{isdA}*) and one probe for a candidate gene that is upregulated in *Staphylococcus aureus* on the skin during colonization, but not reported to be upregulated in blood, for use in an expression clamp strategy (*P_{clfB}*).

[00673] Expression of all genes was normalized to the housekeeping gene *gyrB* (a gyrase subunit) widely used for this purpose in *Staphylococcus aureus*. Ct was

determined by rt PCR. Ct, PCR threshold cycle, is the cycle number at a given fluorescence; the higher the gene (mRNA) quantity, the lower the Ct.

[00674] Preliminary results using serum of a single donor are shown in Table 12.

[00675] Table 12. Effect of Serum exposure on activation of KS promoter candidates in BioPlx-01 and basal expression levels in TSB

Gene	Fold-induction increase in expression in serum treated samples by real time PCR	Basal Expression LeuA/GyrB ratio in TSB
<i>hlgA</i> (gamma hemolysin)	30	0.19
<i>leuA</i> (AA biosynthetic enzyme)	7.7	0.75
<i>sstA</i> (iron transport)	12.8	0.33
<i>sirA</i> (iron transport)	1.2	0.95
<i>isdA</i> (heme transporter)	1.7	0.59
<i>clfB</i> (clumping factor B)	1.3	0.78

[00676] The time course of induction of promoter candidate P_{hlgA} in human serum is shown in FIG. 6 showing a *hlgA/gyrB* ratio in TSB of 0.19, favorable for use in kill switch construct. “no RT”: cDNA made from 75 min timepoint RNA was diluted into a reaction at same dilution as all other samples; if RNA preparation is devoid of gDNA, no signal should be visible. The time course of *sstA* in human serum is shown in FIG. 7 showing *sst/gyrB* ratio in TSB was 0.33. P_{hlgA} and P_{sstA} were selected as preliminary preferred candidates for further evaluation. *hlgA* levels in TSB were only 1/5th of housekeeping gene *gyrB*, or lower, in TSB so this promoter became a lead candidate.

[00677] The experiment was repeated using serum and whole blood from two donors with analysis of total RNA, except that cDNAs were treated with DNaseI to remove contaminating genomic DNA. Specifically, RNA Samples were treated with the turbo DNase kit following the kit protocol for treatment with and inactivation of Dnase.

The “No Reverse Transcription” control (No RT control) – with DNase was at bkg/baseline level, thus acceptable.

[00678] The treated RNA was then used to produce cDNA (and a no RT control was again run). The cDNA was analyzed (starting with *hlga* and *sstA*) by Taqman in with technical triplicates. Results are shown in Table 13.

[00679] Table 13. Promoter Selection- Effect of Serum and Blood exposure on activation of KS promoter Candidates in BioPlx-01 and Basal expression levels in TSB.

Promoter	Fold Induction at 15 min			“Leaky”expression	Serum induction >3 fold through 75 min?
	Serum (donor 1)	Serum (donor 2)	Blood (donor 2)	Target/GyrB (TSB)	
ISDA	83	15	4.1	0.002, 0.022	yes
SSTA	9	6.7	0.3	0.16, 0.333	yes
LEUA	1393	1601	990	0.0013, 0.000017	yes
HLGA	27	6	35	0.23, 0.26	yes
SIRA	5.5	2.6	0.08	0.25, 1.1	no

[00680] P_{isdA} , P_{sstA} , P_{sirA} were eliminated based on data shown in Table 13. P_{sstA} was eliminated because of significant basal expression, and it was not induced in whole blood. P_{sirA} was also eliminated because of significant basal expression, and low magnitude induction in serum, and was not induced in whole blood, as well as exhibiting induction that was not sustained.

[00681] Based on this experiment, P_{leuA} was selected as one preferred promoter because it exhibited very high upregulation in serum, very low basal expression in TSB,

and was not upregulated during colonization. An expression clamp may be employed, but may be optional when using *P_{leuA}* as a promoter. *P_{leuA}* also exhibited strong activation by blood or serum exposure in Malachawa 2011 (microarrays) and in the present example. *leuA* is part of a nine-gene Operon: *ilvDBHC*, *leuABCD*, *ilvA*.. A factor called Cody binds the RR to repress transcription when it is bound to branched chain amino acids (leucine, isoleucine and valine), so when free amino acid levels are above a threshold, the promoter is silent. In porcine *ex vivo* nasal colonization assays with MRSA, amino acid biosynthetic operons including *leu* were not upregulated, and the authors propose that amino acids are present in sufficient quantity during colonization to prevent upregulation of these pathways (Tulinski et al., 2014).

[00682] The gene *leuA* is activated very strongly in blood and serum and has low basal expression, so further understanding is important. *leuA* is within the second of two cassettes in a nine-gene operon; the regulatory region driving it may be immediately upstream of *ilvD* or upstream of *leuA*. One way to understand is to test and compare both variants.

[00683] *ilvDBHC-leuABCD-ilvA*

[00684] *P_{hlgA}* was selected as another preferred promoter because it exhibited high upregulation in serum and blood, and downregulation during nasal colonization. One drawback of *P_{hlg}* is basal expression in TSB; which may be addressed by including an expression clamp for *hlgA*. The peptide HlgA is a subunit of a secreted, pore-forming toxin that lyses host red blood cells and leukocytes. HlgA (class S) associates with HlgB (class F) thus forming an AB toxin in strains producing both gamma-hemolysins and leukocidins (HlgA and LukF-PV can also form a complex).

[00685] Transcription of the HlgA operon is upregulated in TSB by quorum sensing *agr* activation, but *agr* is downregulated in serum while *hlgA* is upregulated, so *hlgA* upregulation is independent of the *agr* pathway in serum. In one paper, the hemolysins were downregulated 5.7 fold compared with TSB during colonization, specifically, porcine nasal explants colonized with MRSA ST398; see Tulinski et al 2014. However, in these experiments, no evidence of expression of *hlgA* was seen during colonization. The regulator *sarT* represses transcription of the hemolysin operon and may be a useful “expression clamp” if *P_{hlgA}* is used to drive the KS, for example by overexpression of *sarT* from a colonization promoter.

[00686] In another embodiment, a synthetic microorganism comprises at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a multiplicity of promoters that are activated by serum or blood, but exhibits little to no expression in human skin, mucosa, or in TSB. There is more certainty of lower expression on skin for *hlgA*, because it is downregulated in colonization. There is more certainly of lower expression in TSB for *leuA*.

[00687] Example 3. Selection of one or more Death Genes

[00688] In this example, cell death gene candidates are evaluated for preparing a synthetic microorganism having at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a first inducible promoter. Relative potencies of death genes are unknown. What appears to be the best death gene is not necessarily the most potent one because of leaky expression. Diversity of mechanism of action could result in killing synergy for two or more death gene combinations. Death gene candidates include: *SprA1*: membrane disruption; *smaI*: genome destruction; and *rsaE*: blocks central metabolism. Various combinations of death genes are shown in Table 14. These plasmids are created and sequenced plasmids for testing of P_{leuA} and P_{hlgA} -driven KS variants.

[00689] Table 14. Death Gene KS Constructs

Strain #	Plasmid name	Promoter	Kill gene (PCD)	Purpose	Comments
1	pTK1	Cadmium inducible	<i>SprA1</i>	+ control shows <i>sprA1</i> SprA1 is a functional kill gene	Cells in TSB treated with Cd should rapidly die
1A	pTK2	Cadmium inducible	<i>sprA1</i> /SprA1 reversed	Neg control	Cells in TSB treated with Cd should NOT rapidly die
2	pTK3	LeuA	<i>sprA1</i> /SprA1	KS	Cells shifted to serum or blood should rapidly die
3	pTK4	LeuA	<i>sprA1</i> /SprA1 reverse	plasmid more	Compare Insertion frequency to # 2

				readily obtainable than # 2	
4	pTK5	LeuA	<i>sprA</i> /SprA1 + CLFB:: <i>sprA</i> /SprA1 as	KS	Expression clamp variant of # 2
5	pTK6	HlgA	<i>sprA</i> /SprA1	KS	Might not be healthy or even obtainable- basal exp
6	pTK7	HlgA	SprA1 + CLFB:: <i>sprA</i> as	KS	Likely healthier than # 5
7	pTK8	Cadmium inducible	<i>smal</i> restriction enzyme	+ control	Cells in TSB treated with Cd should rapidly die
8	pTK9	HlgA	<i>smal</i> restriction enzyme	KS	expression clamp made using antisense
9	pTK10	LeuA	<i>smal</i> restriction enzyme	KS	expression clamp made using antisense
10	pTK11	Cadmium inducible	<i>rsaE</i> sRNA	+ control	Cells in TSB treated with Cd should rapidly die
11	pTK12	HlgA	<i>rsaE</i> sRNA	KS	expression clamp made using antisense
12	pTK13	LeuA	<i>rsaE</i> sRNA	KS	expression clamp does not exist but could be made using antisense

[00690] Death genes may be obtained commercially (Atum) and vector may also be obtained commercially (BEI). Combinations comprising two death genes are constructed after results of single death genes are obtained. Synthetic plasmids, vectors and synthetic microorganisms are prepared based on Table 14.

[00691] Steps in creating a synthetic strain comprising a cell death gene are as follows.

[00692] 1. Produce shuttle vector pCN51 in mid-scale in *E. coli*.

- [00693] 2. Clone death genes into pCN51 in *E. coli* (under Cd-inducible P_{cad}).
- [00694] 3. Replace P_{cad} with serum-responsive promoters; and insert expression clamp where applicable.
- [00695] 4. Verify constructions by sequencing the KS cassettes.
- [00696] 5. Electroporate into *Staphylococcus aureus* RN4220 and select transformants on erythromycin plates (this strain is restriction minus and generates the right methylation pattern to survive in BioPlx-01). RN4220 is a *Staphylococcus aureus* strain used as an intermediate; restriction minus, methylation +; BEI product number NR-45946.
- [00697] 6. Prepare plasmid from RN4220 and restriction digest to confirm ID.
- [00698] 7. Electroporate plasmids into BioPlx-01 and select on erythromycin plates.
- [00699] 8. Synthetic microorganism strains ready for serum experiment.
- [00700] Steps in testing a synthetic microorganism strains having at least one molecular modification comprising a first cell death gene operably linked to a first regulatory region comprising a first promoter are as follows.
- [00701] 1. Growth in TSB plus antibiotic as selective pressure for plasmid.
- [00702] 2. How does growth compare with WT BioPlx-01? Prepare growth curve.
- [00703] 3. Cd-promoter variants: Wash and shift cells to Cd medium (control is WT BioPlx-01 containing empty vector with no death gene).
- [00704] 4. KS variants: Wash and shift cells to serum (control is WT BioPlx-01 containing empty vector with no death gene).
- [00705] 5. Monitor growth using OD_{630 nm} with plate reader (extended period, monitor for appearance of escape mutants).
- [00706] 6. For whole blood test, only perform on winning candidates and use CFU on TSB agar as death readout.
- [00707] 7. If there are apparent escape mutants, shuttle plasmid out to *E. coli* and sequence the whole plasmid.

[00708] Plasmids may be prepared from commercially available products. In one embodiment, pCN51 (6430 bp) is the commercial plasmid for modification. pCN51 is an *E. coli*-SA shuttle vector, with ampR for *E. coli* selection and ermC for *Staphylococcus aureus* selection. This is a pT181 based low copy rolling circle plasmid, containing a Cadmium inducible promoter and BLA terminator. BEI product number NR-46149. Combinations of KS variants are possible in one plasmid. It is possible to insert more than one KS into the MCS of a shuttle vector plasmid.

[00709] 1. The 3 constructs encoding the 3 kill genes are ordered from Atum/DNA2.0, with restriction suites placed strategically at ends of each gene for directional cloning.

[00710] 2. pCN51 shuttle vector (BEI NR-46149), RN4220 *Staphylococcus aureus* (BEI NR-45946), and DC10B *E. coli* (BEI NR-49804) are ordered from BEI Resources.

[00711] 3. The DNA oligonucleotides shown in Table 15 are ordered from for: i) PCR amplification of RRs from BioPlx-01 gDNA, with restriction enzymes at ends for directional cloning, and; ii) DNA sequencing of KS constructs.

[00712] Table 15: Oligonucleotides used for sequencing KS constructs

Oligo Name	Sequence (5' to 3')	Purpose
TKO1	gatgcGCATGCGAAACAGATTATCTATTC (SEQ ID NO: 9)	P _{leuA} PCR Amplification with SphI (upstream pr)
TKO2	gatgcGCATGCCAGATTATCTATTCAAAG (SEQ ID NO: 10)	P _{leuA} PCR Amplification with SphI (upstream pr-alternate)
TKO3	catgatCTGCAGAGTAAATCCCCGTAAATT (SEQ ID NO: 11)	P _{leuA} PCR Amplification with PstI (downstream pr)
TKO4	cacgtgatCTGCAGAGTAAATCCCCGTAAA (SEQ ID NO: 12)	P _{leuA} PCR Amplification with PstI (downstream pr-alternate)
TKO5	gactacGAATTC AGGTGATGAA AAATTTAGAA (SEQ ID NO: 13)	upstream primer to amplify P _{clfB} with EcoRI
TKO6	gactacGAATTC TGATGAA AAATTTAGAACTT	backup to TKO5

	(SEQ ID NO: 14)	
TKO7	cttagctGGATCCAAATATTACTCCATTTC (SEQ ID NO: 15)	downstream primer to amplify P_{clfB} with BamHI
TKO8	cttagctGGATCCAAATATTACTCCATTTCATTTC (SEQ ID NO: 16)	backup to TKO7
TKO9	gatgcGCATGC TCACAACTA TTGCGAAATC (SEQ ID NO: 17)	upstream primer to amplify the P_{hlgA} ; contains SphI
TKO10	gatgcGCATGC AACTA TTGCGAAATC CATTC (SEQ ID NO: 18)	backup to TKO9
TKO11	catgatCTGCAG ATATATAATAATCCATTGT (SEQ ID NO: 19)	downstream primer to amplify P_{hlgA} ; contains PstI
TKO12	catgatCTGCAGATATATAATAATCCATTGTAAAGCG (SEQ ID NO: 20)	backup to TKO11
TKO13	GTGTTACGATAGCAAATGCA (SEQ ID NO: 21)	First sense primer for sequencing constructs containing P_{cad}
TKO14	TTATTGGCTAAGTAGACGCA (SEQ ID NO: 22)	second sense sequencing primer anneals roughly in the middle of the <i>sprA1</i> gene
TKO15	CACATGTTCTTTCCTGCGTT (SEQ ID NO: 23)	primer to anneal just upstream of the serum responsive P_{leuA} and P_{hlgA} . Anneals in the pCN51 vector about 75 nt upstream of the SphI site
TKO16	ACGCGGCCTTTTACGGTTC (SEQ ID NO: 24)	backup for TKO15
TKO17	GAATGGGACTTGTAACGTC (SEQ ID NO: 25)	primer to anneal near the downstream one third of the P_{leuA} because

		its a fairly large segment and TKO15 may not read all the way through
TKO18	GAATGGGACTTGTAACG (SEQ ID NO: 26)	backup for TKO17
TKO19	ATAAACGCCTGCGACCAATA (SEQ ID NO: 27)	primer to anneal near the downstream one third of the P_{hlgA} because its a fairly large segment and TKO15 may not read all the way through
TKO20	GCGACCAATAAATCTTTTAA (SEQ ID NO: 28)	Backup for TKO19

[00713] CLONING

[00714] All gel-electrophoresis agarose gels are 1.0-2.0% agarose in 1X TAE buffer and midori green (Nippon Genetics Europe GmbH) added per the manufacturer's instructions.

[00715] Example 3A. CONSTRUCTING pTK1 and pTK2

[00716] **1. Prepare miniprep quantities of pCN51 and of *sprA1*, *smal*, and *rsaE* plasmids as follows**

- A. Streak the strains on LB+ carbenicillin (100 µg/mL) plates and incubate 15-18 h at 37°C.
- B. Inoculate LB+ carbenicillin (100 µg/mL) liquid with single colony of each and incubate with agitation (240 rpm) for 15-18 h at 37°C.
- C. Prepare 5X replicate minipreps of each strain with Qiagen spin miniprep kit per manufacturer's instructions, elute DNA from each column with 30 µL, and pool the replicate plasmid preps together (freeze DNA at -20°C).

[00717] **2. Digests, ligation, plating**

2.1. Cut pCN51 with PstI and EcoRI to linearize (37°C, 30 mins). Expected size is ~6400 bp (a 35 bp fragment from the multiple cloning site (MCS) is dropped out/not visible on gel).

- 2.2. Cut pCN51 plasmid with Kpn1 and BamHI to linearize (37°C, 30 mins). Expected size is 6400 bp (a 35 bp fragment from the MCS is dropped out).
- 2.3 Cut *sprA1* plasmid from DNA2.0 with Pst1 and EcoRI to liberate the desired 233 bp *sprA1* insert.
- 2.4 Cut *sprA1* plasmid from DNA2.0 with Kpn1 and BamHI to liberate the desired 233 bp *sprA1* insert.
- 2.5. During DNA digestion pour a gel that is 1.5% agarose gel for electrophoresis as described.
- 2.6. Add 8 µL of 6X loading dye to all 4 reactions and to the 1 kb plus DNA size ladder (3 µL in 30 µL).
- 2.7. Run gel at 100 V for 1.5 h.
- 2.8. Excise the bands of interest mentioned above with a clean razor blade.
- 2.9. Melt the slices in 3 volumes of buffer QG from Qiagen gel extraction kit (56°C), vortexing occasionally.
- 2.10. Isolate the paired vector and insert together on one column and elute the material into 30 µl of Qiagen's elution buffer.
 - A. Pst1 + EcoRI insert plus pCN51 Pst1/EcoRI vector.
 - B. Kpn1 + BamHI insert plus pCN51 Kpn1/BamHI.
- 2.11 Set up a waterbath by adding some ice to 500 mL RT water in a styrofoam box; add just enough ice to reach 16°C.
- 2.12. Add 3.4 µL of 10X T4 DNA ligase buffer and mix. Add 1 µL of T4 DNA ligase (4×10^5 U/mL stock from NEB) and incubate for 2 h at 16°C.
- 2.13 Set electroporation unit to 1500 V/ 200 ohms /25 µF.
- 2.14. Thaw 2 vials of DH5α *E. coli* and add 40 µL into each into 2 Eppendorf tubes. Chill 2 electroporation cuvettes on ice.
- 2.15. Add 1 µL of undiluted ligation to 40 µL of the thawed DH5α *E. coli* and transfer to an ice-cold 1 mm gap electroporation cuvette.
- 2.16. Have ready: 1 mL of SOC medium in a 1 mL pipet, sterile 1 mL tips, and 2 sterile 14 mL culture tubes
- 2.17. Electroporate the cells (ligation A first) and then ASAP add 1 mL SOC to the cuvette, pipet up and down 6X, and transfer the whole volume to a fresh 14 mL culture tube for recovery. Repeat this process for electroporation of ligation B. Place the two recovering samples in the shaking water bath at 37°C for 1 h.

- 2.18. Place 2 LB + cabenicillin (100 µg/mL) agar plates inverted with their lids slightly off in the 37°C incubator (not humidified) while the cells recover
- 2.19. After the 1 h recovery period, remove and label the LB + cabenicillin (1050 µg/mL) agar plates accordingly and remove the 14 mL tubes from the waterbath.
- 2.20. Using a sterile glass beads, spread 150 µL of each 1 mL recovery mix onto a plate.
- 2.21 Place the plates in the 37°C incubator for 16-18 h.
- 2.22. Record colony counts for
Ligation A ($P_{cad}::sprA1$ forward) and
Ligation B ($P_{cad}::sprA1$ reverse).

[00718] 3. Screening for positives:

- 3.1 Pick 6 colonies for screening
- 3.2 Inoculate 6 colonies of ligation A and 6 of ligation B, each into 3 mL of liquid LB + cabenicillin (1050 µg/mL) in a 14 mL culture tube..
- 3.3 Shake for 16 h at 37°C.
- 3.4 Isolate plasmid DNAs using Qiagen spin mini kit per manufacturer's instructions, and elute DNA into 40 µL elution buffer.
- 3.5 Digest 5 µL of each of the 12 plasmid DNAs with
 - A. PST1 plus ECOR1
 - B. Kpn1 + BamHI
 - C. Xmn1 alone

Mix for 7 reactions if Pst1+EcoRI. Add 5 µL of DNA solution to 15 µL of digestion mixture and incubate 2 h at 37°C. Do the same for Kpn1 + BamHI and Xmn1 digestions.

[00719] Compare to expected gel patterns: Correct pattern for pTK1 digests: i) EcoRI and PstI; ii) Kpn1 and BamHI; iii) Xmn1. Correct pattern for pTK2 digests: i) EcoRI and PstI; ii) Kpn1 and BamHI; iii) Xmn1.

[00720] Example 3B. Making pTK3 ($P_{leuA}::sprA1$) and pTK6 ($P_{hlgA}::sprA1$) and pTK4 ($P_{leuA}::sprA1$ reversed)

1. Extract gDNA from a log-phase culture of BioPlx-01 using the Qiagen "All prep" kit.
2. Digest pTK1 SprA1 with Sph1 and Pst1 to drop out the cadmium-inducible promoter (P_{cad}).
3. PCR amplify the *leuA* regulatory region (P_{leuA}) from BioplX-01 gDNA using PCR primers that contain the Sph1 restriction sequence upstream and Pst-1 restriction

sequence downstream. (TKO1 and TKO3 Sequences below; or backups TKO2+TKO4). Verify the restriction with gel electrophoresis as previously described.

PCR mixture:

1.0 μ L of gDNA from BioPlx-01 50 ng/ μ L

25.0 μ L dI water

10.0 μ L 5X HF buffer

5.0 μ L 2mM dTNP mix

4.0 μ L primer TKO1 (5 pmol/ μ L stock)

4.0 μ L TKO3 (5 pmol/ μ L stock)

1.0 μ L phusion polymerase NEB

50.0 μ L total

Cycles:

98°C for 2 min

20 cycles of: 98°C 15 sec--64°C 30 sec--72°C 1 min

15 cycles of: 98°C for 15 sec--55°C for 30 sec--72°C for 1 min

Hold: 4°C, indefinitely

4. PCR amplify the *hlgA* regulatory region (*P_{hlgA}*) from BioplX-01 gDNA using PCR primers that contain the Sph1 restriction sequence upstream and Pst-1 restriction sequence downstream. (TKO9 and TKO11 or backup set TKO10 or TKO12). PCR conditions are as above for *P_{leuA}* except for the identity of the primers.

5. Using the Qiagen PCR cleanup kit, clean the PCR reactions and elute into 43 μ L of elution buffer

6. Cut the *P_{leuA}* PCR product from step 3 and the *P_{hlgA}* PCR product from step 4 with Sph1 and Pst1. Do this by adding 5 μ L of 10X CutSmart (NEB) and 1 μ L each of Sph1 and Pst1 and incubating for 2 h at 37°C.

7. Digest pTK1 with Sph1/Pst1.

8. Fractionate the pTK1 Sph/Pst digest and the Sph/Pst digested *P_{leuA}* and *P_{hlgA}* on a 1.5% agarose gel and excise the ~6000 pTK1 backbone and the *P_{leuA}* (390 bp) and *P_{hlgA}* (253 bp) fragments with a clean razor blade.

9. Divide the pTK1 backbone slice in two and combine one half with the LeuA slice and the other half with the HlgA slice. Melt together and isolate together using the Qiagen gel extraction kit. Elute each into 30 μ L EB.

10. Add 3.4 μ L of 10X T4 DNA ligase buffer and 1 μ L of T4 DNA ligase and incubate at 16°C for at least 1 h.
11. Follow steps in section 2.13-2.22 for electroporation, recovery, and colony plating.
12. The two ligations aim to generate $P_{leuA}::sprA1$ wt in the forward orientation (pTK3) and $P_{hlglA}::sprA1$ wt in the forward orientation (pTK6).

[00721] Example 3C. Making pTK4 (conduct steps concurrently with pTK3)

1. Extract gDNA from a log-phase culture of BioPlx-01 using gDNA isolation kit.
2. Digest pTK2 (sense *sprA1*) with Sph1 and Pst1 to drop out the P_{cad} (see above for digestion conditions).
3. Insert the Sph1/Pst1 digested P_{leuA} fragment from above into the Sph1/Pst1 digested pTK2 to generate $P_{leuA}::sprA1$ wt in the reverse orientation (pTK4). Details of the gel extraction, ligation and electroporation processes are the same as in Section 2 of cloning above.

[00722] Screening pTK3, pTK4 and pTK6:

- 3.1 Pick 6 colonies of each ligation for screening
- 3.2 Inoculate 6 colonies of pTK3 and 6 of pTK4 and 6 of pTK6 each into 3 mL LB + cabenicillin (100 μ g/mL) in 14 mL culture tubes.
- 3.3 Incubate with agitation for 16 h (37°C, 240 rpm).
- 3.4 Isolate plasmid DNA using a mini prep kit and elute DNA with 40 μ L elution buffer.
- 3.5 Digest 5 μ L of each of the 18 plasmid DNAs as follows (prepare enough digestion reaction mixture for 20 reactions to account for pipetting errors):

A. Sph1 plus Pst1

B. Xmn1.

Add 5 μ L DNA to 15 μ L digestion reaction mixture and incubate 2 h at 37°C

- 3.6 Verify digestion with gel electrophoresis, compare to expected gel patterns for pTK3, pTK4, and pTK6.

[00723] Making pTK5 and pTK7

1. Use gDNA of BioPlx-01 prepared above.
2. PCR amplify the *clfB* RR (P_{clfB}) from BioPlx-01 genomic DNA using primers with a EcoRI restriction sequence upstream and BamHI restriction sequence downstream (primers: TKO5 and TKO7)

PCR Mixture (50 μ L total volume)

- 1.0 μ L of gDNA from BioPlx-01 50 ng/ μ L
- 25.0 μ L dI water
- 10.0 μ L 5X HF buffer (NEB)
- 5.0 μ L 2mM dTNP mix
- 4.0 μ L primer TKO5 (5 pmol/ μ L stock)
- 4.0 μ L TKO7 (5 pmol/ μ L stock)
- 1.0 μ L phusion polymerase (NEB)

Cycles:

98°C for 2 min

20 cycles of: 98°C 15 sec--64°C 30 sec--72°C 1 min

15 cycles of: 98°C for 15 sec--55°C for 30 sec--72°C for 1 min

Hold: 4°C, indefinitely

3. Use 5 μ L of the PCR reactions for gel electrophoresis as previously described.
4. Using the PCR cleanup kit, clean the PCR reaction and elute with 30 μ L of elution buffer.
5. Digest the P_{clfB} PCR product with BamH1 and EcoR1 and insert it into the EcoR1/BamH1 digested pTK3 backbone to generate pTK5. This plasmid will contain *sprA1* regulated by P_{leuA} and the *sprA1*_{AS} regulated by P_{clfB} . Using the same P_{clfB} fragment, insert it into the EcoR1/BamH1 digested pTK6 to generate pTK7. This plasmid will contain *sprA1* regulated by P_{hlga} and the *sprA1*_{AS} regulated by P_{clfB} SprA1. Details of the gel extraction, ligation and electroporation processes are the same as in section 2 of cloning above.

[00724] Screening for pTK5 and pTK7

- 3.1 Inoculate 6 colonies of ligation pTK5 and 6 colonies of ligation pTK7 into 3 mL LB + cabcenicillin (100 μ g/mL) in 14 mL culture tubes.
- 3.3 Incubate with agitation for 16 h (37°C, 240 rpm)
- 3.3 Isolate plasmid DNA using a mini prep kit and elute DNA into 40 μ L elution buffer.
- 3.5 Digest 5 μ L of each of the 12 plasmid DNAs with:

A. BamHI + EcoRI

B. Xmn1 alone

Prepare digestion reaction mixture with BamHI/EcoRI and XmnI following the manufacturer's suggestions.

Add 5 µL of plasmid solution to 15 µL of digestion reaction mixture and incubate for 2 h at 37°C. Verify the digestion with gel electrophoresis as previously described.

[00725] Example 3D. Constructing pTK8, pTK9 and pTK10 (P_{cad} -*smal*, P_{hlga} -*smal* and P_{leuA} -*smal* respectively).

The *smal* gene was ordered from DNA2.0 with a PstI restriction site upstream and EcoRI restriction site downstream to allow for insertion into the following:

- pCN51 to make $P_{cad}::smal$ resulting in pTK8
 - pTK6 from which *sprA1* has been removed with PstI/EcoRI to make P_{hlga} -*smal* resulting in pTK9
 - pTK3 from which *sprA1* has been removed with PstI/EcoRI to make P_{leuA} -*smal* resulting in pTK10
1. Digest pCN51, pTK6 and pTK3 with PstI and EcoRI by *sprA1*. incubating each for 2 h at 37°C.
 2. Generate the *smal* fragment by digesting the ordered plasmid containing the gene with PstI and EcoRI (2 h at 37°C). Verify the digestion with gel electrophoresis (expected fragment size is 757 bp).
 3. Follow steps 2.5 to 2.22 for gel extraction, ligation, electroporation, recovery, and antibiotic selection.

[00726] Screening for pTK8, pTK9, and pTK10

3.1 Inoculate 6 colonies of ligation pTK8 and 6 colonies of ligation pTK9 and 6 colonies of ligation pTK10 into 3 mL LB + cenicillin (100 µg/mL) in 14 mL culture tubes.

3.3 Incubate with agitation for 16 h (37°C, 240 rpm)

3.3 Isolate plasmid DNA using a mini prep kit and elute DNA into 40 µL of elution buffer

3.5 Digest 5 µL of each of the 12 plasmid DNAs with

- A. PstI and EcoRI
- B. SphI and XcmI
- C. XmnI alone

Follow previously described restriction reaction and gel electrophoresis procedures.

[00727] Example 3E. Making pTK11, pTK12 and pTK13 (P_{cad} -*rsaE*, P_{hlga} -*rsaE* and P_{leuA} -*rsaE* respectively).

The *rsaE* gene was ordered from DNA2.0 with an upstream PstI restriction site and a downstream EcoRI restriction site to allow for insertion into the following plasmids:

- pCN51 to make P_{cad} -*rsaE* resulting in pTK11
- pTK6 from which *sprA1* has been removed with PstI/EcoRI restriction to make P_{hlxA} -*rsaE* resulting in pTK12
- pTK3 from which *sprA1*/SprA1 has been removed with PstI/EcoRI restriction to make P_{leuA} -*rsaE* resulting in pTK13

1. Digest pCN51, pTK6 and pTK3 with PstI and EcoRI *sprA1* as described in previous sections.

2. Digest ordered DNA containing *rsaE* PstI and EcoRI following manufacturer's suggestions.

Verify digestion with gel electrophoresis (. *rsaE* fragment should be 142 bp).

3. Follow steps 2.5 to 2.22 for gel isolation, ligation, electroporation, recovery, and antibiotic selection.

Example 4. Production of *sprA1* Clamp and no clamp constructs using DNA2.0 to make inserts:

[00728] Here pCN51 is employed as the vector backbone because it has cadmium inducible promoter (P_{cad}), Bla terminator, ampicillin resistance for *E. coli* and erythromycin resistance for *Staphylococcus aureus*. In Drutz 1965, 502a was shown to be sensitive to 2 µg/mL erythromycin.

[00729] Plasmid pTK1: Positive control cassette to prove that *sprA1*, when induced, causes death.

[00730] 1. Order the following insert from DNA2.0. It is cut out of the ordered vector with PstI and EcoRI restriction enzymes, and inserted into PstI/EcoRI-digested pCN51. It is just the open reading frame and a little flanking downstream to capture *sprA1*-essentially as in Sayed et al. 2012, except that the P_{cad} feature is used instead of the aTc promoter (P_{tet}). This sequence was verified in pDRAW, to assure strategy will work.

CTGCAG~~ggtaccgcagagaggaggt~~tataaggtg
ATGCTTATTTTCGTTCACATCATAGCACCAGTCATCAGTGGCTGTGC
 CATTGCGTTTTTTTCTTATTGGCTAAGTAGACGCAATACAAAA~~TAGGTGA~~
 CATATAGCCGACCAATAAAAAATCCCCTCACTACCGCAAATAGTGAGGGG
 ATTGGTGTataagtaaataactattttcgttgt
~~ggatccttgact~~*GAATTC* SEQ ID NO: 122

Resulting plasmid: pTKXXX

Underlined upper case: start codon

Italicized: stop codon

BOLD: PstI site upstream

UPPERCASE BOLD ITALICIZED: EcoRI site

lower case bold italicized: KpnI site

Rust color: shine-delgarno (naturally used for SprA1)

Lower case underlined: BamHI site

[00731] Produce pTK2: Reverse the insert in pTK1

[00732] 1. Cut the insert of pTK1 out with Kpn1 and BamHI and insert it into Kpn1 and BamHI-digested pCN51. This creates the antisense orientation of the toxin gene and toxin should not be expressed at all, whether it is induced with cadmium or not. Product is pTK2.

[00733] PTK3 and PTK4: *P_{hlgA}* regulating *sprA1* toxin to prove that *sprA1*, when induced by serum or blood, causes cell death (forward and reverse constructs, respectively).

[00734] *sprA1_{AS}* is present but has only its natural promoter, so the expression clamp should be inactive – and also if *P_{hlgA}* is leaky, some cell toxicity may occur because the expression clamp is not present.

[00735] pTK3:

1. Digest pTK1 (sense *sprA1*) with Sph1 and Pst1 to drop out *P_{cad}*.
2. PCR amplify the *hlgA* regulatory region (*P_{hlgA}*) from strain 502a using PCR primers that contain an upstream Sph1 restriction site and Pst1 downstream restriction site. (Primers: TKO1 and TKO2)
3. Cut the *P_{hlgA}* PCR product with Sph1 and Pst1 and insert it into the Sph1/Pst1 digested pTK1 to generate *P_{hlgA}::sprA1* wt in the forward orientation generating pTK3.

1 TTGCGAAATC CATTCTCTT CCACTACAAG CACCATAATT AAACAACAAT
 AACGCTTTAG GTAAGGAGAA GGTGATGTTT GTGGTATTAA TTTGTTGTTA

 51 TCAATAGAAT AAGACTTGCA AAACATAGTT ATGTCGCTAT ATAAACGCCT
 AGTTATCTTA TTCTGAACGT TTTGTATCAA TACAGCGATA TATTTGCGGA

 101 GCGACCAATA AATCTTTTAA ACATAACATA ATGCAAAAAC ATCATTTAAC
 CGCTGGTTAT TTAGAAAATT TGTATTGTAT TACGTTTTTG TAGTAAATTG

 151 AATGCTAAAA ATGTCTCTTC AATACATGTT GATAGTAATT AACTTTTAAC
 TTACGATTTT TACAGAGAAG TTATGTACAA CTATCATTA TTTGAAAATTG

 201 GAACAGTTAATTCGAAAACGCTTACAAATGGATTATTATATAT SEQ ID NO: 327
 CTTGTCAATT AAGCTTTTGC GAATGTTTAC CTAATAATATATA SEQ ID NO: 328

[00736] TKO1: 5'-gatgcGCATGCTTGC GAAATC CATTCTCTT-3' (contains SphI)

SEQ ID NO: 329

[00737] TKO2: 5'-catgatCTGCAGATATATAATAATCCATTTGTAAGCG-3' (contains PstI) SEQ ID NO: 20

[00738] pTK4:

1. Digest pTK2 (reverse *sprA1*) with SphI and PstI to drop out the cadmium promoter.
2. Insert the same SphI/PstI digested *P_{hlgA}* PCR product. This provides the reverse orientation *SprA1*.

[00739] pTK5: Expression clamp for pTK3, using *P_{clfB}* to drive *SprA1_{AS}*

1. PCR amplify the *P_{clfB}* from 502a gDNA using with primers to generate an upstream EcoRI restriction site and a BamHI downstream restriction site.
2. Digest pTK3 with EcoRI and BamHI and insert the EcoRI/BamHI-digested *P_{clfB}*.

[00740] The resulting plasmid is called pTK5 and will contain the *SprA1* sense regulated by the serum responsive *P_{hlgA}* (upregulated) and the *sprA1_{AS}* *SprA1* regulated by serum responsive *P_{clfB}* (downregulated).

[00741] The sequence below is the P_{clfB} (219 nucleotides immediately upstream of TTG start codon).

1 AGGTGATGAA AAATTTAGAA CTTCTAAGTT TTTGAAAAGT AAAAAATTTG
 TCCACTACTT TTAAATCTT GAAGATTCAA AAACTTTCA TTTTAAAC
 51 TAATAGTGTA AAAATAGTAT ATTGATTTTT GCTAGTTAAC AGAAAATTTT
 ATTATCACAT TTTTATCATA TAACTAAAAA CGATCAATTG TCTTTTAAAA
 101 AAGTTATATA AATAGGAAGA AAACAAATTT TACGTAATTT TTTTCGAAAA
 TTCAATATAT TTATCCTTCT TTTGTTTAAA ATGCATTAAA AAAAGCTTTT
 151 GCAATTGATA TAATTCTTAT TTCATTATAC AATTTAGACT AATCTAGAAA
 CGTAACTAT ATTAAGAATA AAGTAATATG TTAAATCTGA TTAGATCTTT
 201 TTGAAATGGA GTAATATTT SEQ ID NO: 129
AACTTTACCT CATTATAAA SEQ ID NO: 130

[00742] Primer: 5'--gactacGAATTC AGGTGATGAA AAATTTAGAA-3' SEQ ID NO: 13

[00743] Primer: 5' cttagctGGATCCAAATATTACTCCATTTCAA-3' SEQ ID NO: 15

[00744] PepA1 (SA newman)

MQGFKEKHQELKKALCQIGLMRSISEVKQLNIA SEQ ID NO: 113

[00745] pTK6. serum responsive promoter 2--*SprA1*

[00746] In this construct, the responsive promoter 2 is P_{leuA} .

1. Digest pTK1 (containing sense *sprA1*) with Sph1 and Pst1 to drop out P_{cad} .
2. PCR amplify P_{leuA} from *Staphylococcus aureus* 502a gDNA using PCR primers that contain an upstream Sph1 restriction site and a downstream Pst1 restriction site (Primers:TKO5 and TKO6).
3. Digest the P_{leuA} PCR product with Sph1 and Pst1 and insert it into the Sph1/Pst1 digested pTK1 to generate $P_{leuA}::SprA1$ wt in the forward orientation generating pTK6.

[00747] In *Staphylococcus aureus*, the *ilvleu* operon consists of *ilvDBHC-leuABCD-ilvA* (9 genes). It is the BCAA biosynthetic operon.

[00748] Example 5. Preparation of Electrocompetent DC10B

[00749] Electrocompetent bacteria are prepared by harvesting log-phase cells and washing the cells extensively in sterile de-ionized water to lower the conductivity and to render the cells into an appropriate osmotic state for the electroporation process.

[00750] 1. From freshly streaked antibiotic free plates, inoculate 250 mL LB media with each strain and incubate with agitation (37°C , 240 rpm)..

[00751] 2. Turn on centrifuge and cool rotor to 4°C well in advance of harvesting cells. Place 1 L of sterile filtered 10% glycerol on ice well in advance of harvesting cells.

[00752] 3. Monitor growth by OD₆₃₀ and when the cells are at 1.0 OD₆₃₀ units per mL, place flask immediately on wet ice for 10 minutes. From this point on the cultures must be kept ice cold. Pour each 250 mL culture into chilled 500 mL sterile centrifuge bottles.

[00753] 4. Centrifuge (15 mins, 3500 rpm, 4°C). Pour off the supernatant and aspirate any residual broth.

[00754] 5. Add 250 mL of sterile 10% glycerol to each of the centrifuge bottles and completely suspend the cells by pipetting up and down.

[00755] 6. Repeat 4 and 5 two more times.

[00756] 7. Pour off the supernatant and suspend the cells in 2 mL 10% glycerol by pipetting up and down.

[00757] 8. To freeze, aliquot 100 µL of the culture to microcentrifuge tubes on wet ice. Once you have used all of the culture, transfer the tubes to a dry ice/ethanol bath for 10 minutes. Once the cultures are frozen, transfer cells to a -80°C freezer for storage.

[00758] To confirm cell's efficiency – transform cells with 1 µL of pUC19 (10pM).

[00759] Electroporation conditions for *E. coli* are 1500 V, 25 µF, 200 ohms. Use 1 µL of plasmid miniprep from DH5α and electroporate it into 50 µL of the electrocompetent DC10B.

[00760] 1. Electrocompetent *E. coli* are thawed on ice, and 1 µL of plasmid is added to 50 µL of cells in an ice cold 0.1 cm gap electroporation cuvette.

[00761] 2. Electroporate as above and add recovery medium immediately (1 mL, SOC medium).

[00762] 3. Agitate at 37°C for 1 h at 250 rpm and plate 100 µL onto LB+100 µg/mL carbenicillin. Incubate plates for 16 h at 37°C.

[00763] Example 6. SATransformation

[00764] Techniques for transformation are adapted from Chen, W., et al. 2017, Rapid and Efficient Genome Editing in *Staphylococcus aureus* by Using an Engineered CRISPR/Cas9 System. J Am Chem Soc 139, 3790-3795. Materials to have on hand: LB agar plate containing 50 µg/ml kanamycin; sequencing primers for PCR screening of 12 clones; TSB broth with kanamycin, sterile tubes for bacterial growth; PCR reagents to do colony PCR (master mix for 500 µl) and PCR grade H₂O.

[00765] 10 µL product of Golden Gate assembly is transformed into 100 µL *E. coli* DH10B competent cells. The successful colonies are selected on a LB agar plate containing 50 µg/mL kanamycin. The success for the construction of the pCasSA-NN_spacer plasmid was verified by PCR or sequencing.

[00766] Example 7. Purification plasmids from *E. coli* DH10B to confirm sequence

[00767] 1. DNA sequencing of inserts

[00768] Primers TKO13 through TKO20 are used variously to sequence the inserts of these 13 plasmids. The primers to use for each plasmid are indicated in Table 15. The kill gene inserts are obtained from DNA2.0. PCR amplified *P_{leuA}* and *P_{hlga}* promoters to evaluate any possible polymerase errors for these fragments.

[00769] 2. Assembly and confirmation of sequences

[00770] 2.1 Raw chromatograms are inspected and only high quality regions (very high signal/noise and good peak separation) are chosen to use in assembly process.

[00771] 2.2 Overlap regions of sequence reads from successive primers are identified and removed; unique reads are strung head to tail in Microsoft word with color coding of the text.

[00772] 2.3 Clustal W is used to generate sequence alignments of theoretical sequences to the actual. Any discrepancies are confirmed by manual inspection of chromatograms.

[00773] Example 8. Preparation of Electrocompetent BioPlx-01 and RN4220

[00774] Electrocompetent bacteria are prepared by harvesting log phase cells and washing the cells extensively in sterile de-ionized water to lower the conductivity and to render the cells into an appropriate osmotic state for the electroporation process.

[00775] Materials to have on hand:

[00776] 1. 500 mL orange capped v-bottom corning centrifuge bottles

[00777] 2. 50 mL falcon tubes

[00778] 3. 1.5 mL sterile microcentrifuge tubes

[00779] 4. 96 well plate for A630 measurements

[00780] 5. 10 and 25 mL sterile pipets and sterile pipet tips all sizes

[00781] 6. TSB broth (need 600 mL total)

[00782] 7. 1L of Sterile 500 mM sucrose on wet ice well in advance of harvesting cells

[00783] Protocol

[00784] 1. From freshly streaked antibiotic free plates, inoculate 250 mL TSB media with each strain and incubate with agitation (37°C, 250 rpm)..

[00785] 2. Turn on centrifuge and cool rotor to 4°C well in advance of harvesting cells. Place 1 L of 10% glycerol on ice well in advance of harvesting cells.

[00786] 3. Monitor growth by OD₆₃₀ and when the cells are at 1.0 OD₆₃₀ units per mL, place flask immediately on wet ice for 15 min. From this point on the cultures must be kept ice cold. Pour each 250 ml culture into chilled 500 ml sterile centrifuge bottles.

[00787] 4. Centrifuge at 2900 rpm for 15 min. Pour off the supernatant and aspirate any residual broth.

[00788] 5. Add 250 ml of 10% glycerol to each of the centrifuge bottles and completely suspend the cells by pipetting up and down.

[00789] 6. Centrifuge at 2900 rpm for 15 min. Pour off the supernatant, it is not necessary to aspirate. Completely suspend the cells in 250 ml glycerol and re-centrifuge.

[00790] 7. Pour off the supernatant and suspend the cells in the residual glycerol by pipetting up and down.

[00791] 8. To freeze, add 100 microliters of the culture to microcentrifuge tubes on wet ice. Once you have used all of the culture, transfer the tubes to a dry ice/ethanol bath for 10 minutes. Once the cultures are frozen, transfer them to a -80°C freezer.

[00792] **Example 9. Design and test CRISPR gRNA sequences and test pCasSA simultaneously**

[00793] In this example a CRISPR-Cas system is obtained that is effective in *Staphylococcus aureus* (pCasSA) from Addgene (Addgene plasmid repository, Cambridge, MA), identify an intergenic region to target from prior experiments, and finally, design and test gRNA aimed for the intergenic region.

[00794] 1. Order verified CRISPR components from Addgene as shown in Table 16.

[00795] Table 16. CRISPR Plasmids

ID	Plasmid	Gene/Insert	Vector Type
42876	pCas9	tracr/Cas9	Bacterial Expression, CRISPR; E.coli
42875	pCRISPR	CRISPR-BsaI	Bacterial Expression, CRISPR; E.coli
65770	BPK2101	CRISPR-Cas9	Bacterial expression plasmid for <i>Staphylococcus aureus</i> Cas9 & sgRNA (need to clone in spacer into BsaI sites): T7-humanSaCas9-NLS-3xFLAG-T7-BsaIcassette-Sa-sgRNA
98211	pCasSA	CRISPR-Cas9	Sa-specific CRISPR

[00796] 2. Select CRISPR gRNA target sites. Find where to target, this should be in an intergenic region so as not to disrupt viability. Currently, one such region has been identified between 1,102,100 and 1,102,700 bp in the 502a genome, GenBank: CP007454.1, as shown in FIG. 8. This region aligns with the region previously identified in the recombinant approach.

[00797] 3. Once region has been chosen, use CRISPRScan (<http://www.crisprscan.org/>) Moreno-Mateos et al., 2012, Nature Methods 12, 982-988, to find putative gRNAs as shown in FIG. 9; note that the usable sequence is in all caps.

[00798] 4. Check for possible off-target binding using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=MicrobialGenomes_1280&DB_GROUP=AllMG) or searching the sequence directly (APE or similar). Note: gRNA marked as non-canonical will often have a single mismatched base pair, these will likely still work but may cause additional off target effects

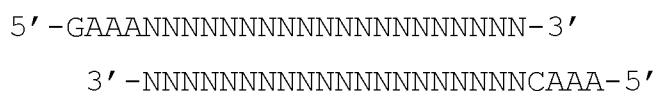
[00799] 5. Modify and order oligos as shown in Table 4B, FIG. 4A-4D. Name of oligos is shown in the format = oligo#, BPC (BioPlx CRISPR), Target #, direction (FOR or REV), followed by the target sequence.

[00800] 6. Add each of the CRISPR targeting sequences into the pCasSA plasmid as per protocol shown below, adapted from Chen, W. et al. 2017. *Rapid and Efficient Genome Editing in Staphylococcus aureus by Using an Engineered CRISPR/Cas9 System*. J Am Chem Soc 139, 3790-3795.

[00801] a. Oligo Design

[00802] Select a 20 bp-spacer sequence before NGG (NGG is not included in the spacer) in the target gene of *Staphylococcus aureus* (40%~60% GC ratio is the best). Synthesize the two oligos in the following form (described above):

[00803] Note: FOR primer should be immediately upstream of the NGG in the target sequence.



[00804] b. Phosphorylation

[00805] Prepare phosphorylation mixture as shown in Table 17.

[00806] Table 17. Phosphorylation mixture

2 μ l	oligo I (50 μ M) _[SEP]
2 μ l	oligo II (50 μ M) _[SEP]
5 μ l	10x T4 DNA ligase buffer (NEB)
1 μ l	T4 polynucleotide kinase (Takara)

40 μ l	ddH ₂ O
50 μ l	total

[00807] Incubate at 37 °C for 1 hour.

[00808] c. Annealing

[00809] Add 2.5 μ l of 1 M NaCl to the phosphorylated oligo pairs. Incubate at 95 °C for 3 min and slowly cool down to room temperature (use a thermocycler). (Alternatively, use a heat block and take the block out of the heater and let it cool naturally for 2 hours.) Dilute the annealed oligos 20 times using ddH₂O.

[00810] d. Vector Digestion

[00811] Digest 1-2 μ g of pCas9 with BsaI (NEB) as shown I Table 18.

[00812] Table 18. Vector digestion mixture

x μ l (1-2 μ g)	pCas9
1 μ l	BsaI (NEB)
5 μ l	10 x NEB Buffer
0.5 μ l	100X BSA
y μ l (to 50 μ l)	ddH ₂ O
50 μ l	total

[00813] Gel purify digested pCas9 (important for successful cloning).

[00814] e. Ligation

[00815] Prepare ligation mixture as shown in Table 19.

[00816] Table 19. Ligation mixture

1 μ l (possibly more)	Gel purified, BsaI digested Cas9
2 μ l	Diluted oligos
2 μ l	10x T4 ligase buffer
1 μ l	T4 ligase
x μ l (to 20 μ l)	ddH ₂ O
20 μ l	total

- [00817] Incubate at RT for 2h or 16C for O/N.
- [00818] Transform into *E. coli* cells (DH5a, DH10B or DC10B).
- [00819] f. Select for plasmid uptake
- [00820] Select for plasmid uptake by plating cells on LB-agar plates with Kanamycin (50 ug/mL). Note: The pCasSA plasmid causes the *E. coli* to grow very slowly at 30° C and plates may need to be incubated for 24-36 hours in order to see colonies.
- [00821] Once colonies are visible select a few for liquid grow up in LB broth with Kanamycin (50 ug/mL). Save an aliquot of liquid culture for easier grow up at a later date.
- [00822] In a cryotube, add 50% sterile glycerol to liquid culture mix by inverting, then place at -80° C for long term storage.
- [00823] Extract the plasmids using Qiagen kit, spec and store and -20° C.
- [00824] 7. Verification of inclusion by PCR and/or sequencing
- [00825] a. PCR Testing
- [00826] Test using 21BPC FOR (SEQ ID NO: 63) and 22BPC REV (SEQ ID NO: 64) on the templates generated above in step 6.
- [00827] Perform PCR of constructs. The PCR products will be ~ 275 bp in the uncut pCasSA vector (positive control = intact pCasSA vector). PCR using the digested pCasSA vector should not produce any products (negative control = BsaI digested pCasSA vector).
- [00828] A small portion of the digested product should be tested to ensure 100% efficacy. Testing can be by PCR or gel electrophoresis directly on the digested plasmid. PCR on the pCasSA vector with the gRNA sequences will produce ~278 bp amplicons. Note: these will not be visibly different when compared to the intact pCasSA vector. As such, the BsaI digestion needs to be 100%.
- [00829] b. Sequencing method
- [00830] Prepare the PCR products generated above for sequencing. Clean up PCR reaction using spin column clean up kit per manufacturers protocol.
- [00831] Measure concentration of purified PCR product using NanoDrop.
- [00832] Mix sample with either forward or reverse primer (21BPC FOR and 22BPC REV, respectively) for sequencing with Quintara Biosciences.

[00833] PCR product at 5 ng/ul and primer at 5 pmol/ul (5 uM). PCR products from the intact pCasSA vector should be sequenced alongside the other products to provide a baseline.

[00834] 8. Testing CRISPR-Cas efficacy/targeting

[00835] Introduction of any plasmid with the inserted gRNA sequences should cause a double-strand break at the targeted CRISPR site. Additionally, the lack of a homologous sequence for homology directed repair (HDR) will cause double strand break induced lethality. Therefore, transforming the targeting plasmids with the targeted plasmid should result in a death rate corresponding to the CRISPR targeting efficacy.

[00836] Transform each of the 10 (assuming all targeting combinations worked) into separate aliquots of electrocompetent RN4220 *Staphylococcus aureus* cells.

[00837] In this case targets 1, 4, and 6-10 should show activity in the RN4220 cells (the sequences are similar enough to allow CRISPR gRNA binding).

[00838] **Example 10. Design and test homology dependent repair templates and efficacy using a fluorescent reporter controlled by a constitutive promoter**

[00839] a. Homologous arms are designed of varying length (200, 300 and 400 bp) corresponding to the ~600 bp intergenic region identified above. For proof of viability, a fluorescent reporter gene (e.g., mCherry) is inserted under control of a constitutive promoter (rspL). The promoter and reporter will be flanked with restriction sites (Not1 and Xma1) to allow transgene swapping. The current design contains a single stop codon. Optionally additional stop codons may be added. Constructs are designed and ordered through ATUM (formerly DNA2.0). This entire sequence (homologous arms + promoter + mCherry) is placed into the pCasSA vector using the Xho1 and Xba1 restriction sites.

[00840] b. Checking for mCherry incorporation/expression

[00841] Once the full pCasSA-XX-XXX vector is assembled and transformed into an *Staphylococcus aureus* strain, verify: 1) mCherry expression, and 2) genomic incorporation of the mCherry sequence. We currently have a few viable methods to check for these. Note: mCherry expression should occur in bacteria that maintain the plasmid as well as those with successful incorporation. To differentiate these, the plasmid must be cured (removed), except in the case of PCR which may be able to differentiate between the two.

- [00842] For Plasmid curing (with repF cassette):
- [00843] Grow a liquid culture at 30 °C with antibiotic as previous;
- [00844] Dilute 3-5 ul of this culture 1000-fold in fresh TSB (no antibiotic);
- [00845] Place at 42-43 °C until growth is apparent (e.g., overnight).
- [00846] Streak the liquid culture on TSA plates with and without chloramphenicol and grow at 37 °C.
- [00847] Cultures should grow on - chlor plate and should not on +chlor plate at 37 °C, if so, the plasmid has been removed
- [00848] For Fluorescence microscopy:
- [00849] The mCherry fluorophore is excited by ~587 nm light and emits ~610 nm.
- [00850] For PCR:
- [00851] PCR across the inserted region to confirm incorporation. Primers designed to amplify: Across the insertion region (41/42 and 43/44). To test for the presence of mCherry (51/45). To verify the presence of genomic DNA (TKO 1/3). Mixing and matching insertion and mCherry primers can also serve to test for mCherry incorporation.
- [00852] Incorporation may also be confirmed by Western blot analysis.
- [00853] Employ western blot equipment: gel box and iBlot transfer system. Employ Primary anti-mCherry antibody, Secondary colorimetric antibody, Precast gels (or gel casting equipment and reagents), iBlot transfer kits, Protease inhibitors, Protein extraction solutions (e.g., RIPA), Protein markers (ladders), and Buffers (TBS, tween etc.) as known in the art.
- [00854] **Example 11. Analysis of KS promoters with fluorescent reporters**
- [00855] The fluorescent reporter is under control of the promoters identified in the recombinant approach (*PleuA*, *PhlgA* etc). This combination allows testing of the efficacy of the chosen promoter with a measurable (positive) outcome. Preferably, the mCherry would be placed in the constructs based on the pCN51 backbone. . The combination is used to test for multiple possible issues:
- [00856] If the plasmid containing cells are exposed to blood/serum mCherry should be expressed. This can be verified either with fluorescence microscopy (Ex 587 nm, Em 610 nm) or by western blotting for the mCherry protein.
- [00857] If the mCherry protein is created in “normal” conditions (no blood/serum activation) then the promoter is “leaky”. Leaky activation could explain some of the

issues obtaining KS plasmids with certain promoters (i.e. P_{leuA}) as even low levels of KS expression could cause a loss of viability.

[00858] What is the rate and conformity of the upregulation caused by a specific primer?

[00859] Cells are viewed in real time (fluorescence microscopy) or through time course sampling (western blot) to observe the rate of fluorescence generation upon exposure to blood and/or serum.

[00860] **Example 12. Insertion of KS into BioPlx-01, verify incorporation, test for efficacy and longevity**

[00861] A KS of choice is inserted in a pCasSA vector using NotI and XmaI restriction sites flanking each sequence. The pTK is amplified using primer BP-40 which adds the XmaI restriction site. The KS is inserted into *Staphylococcus aureus* 502a cells and genomic incorporation verified. The incorporated cells are cured of the plasmid and tested for KS activity when exposed to blood/serum. The KS cells named "BioPlx-XX" are then passaged as described herein to analyze longevity and viability.

[00862] **Example 13. Confirm/characterize the rate and extent of serum-induced cell death.**

[00863] The KS cells BioPlx-XX having the KS are grown side-by-side with BioPlx-01 (*Staphylococcus aureus* 502a WT) in TSB, and then washed and shifted to fresh human serum. The KS strain will "flatline" soon after the shift whereas the WT strain will begin to grow in the serum.

[00864] **Example 14. Evaluate the stability of the KS strain BioPlx-XX**

[00865] This experiment is performed to demonstrate that the KS in BioPlx-XX is phenotypically and genotypically stable during *in vitro* propagation.

[00866] Phenotypic stability (in this case, KS performance) will be assessed by determining the rate of cell death in serum after passaging the strain for X, Y and Z generations, where X is the number of doublings experienced in strain manufacturing to produce a single clinical lot of material sufficient to treat 200 patients, and Y and Z are the number of generations experienced after up to 41 total culture doublings. We are aiming for 4×10^9 cells per patient X 200 patients = 8×10^{11} total cells.

[00867] A dose of 4×10^9 cells per patient X 200 patients = 8×10^{11} total cells.

- [00868] 1. Inoculate 5 mL of TSB with a single large colony of BioPlx-01 and a second 5 mL of BioPlx-02 - both have been streaked from the frozen master cell banks. (approximate density is 0.05 A630/mL).
- [00869] 2. Allow the 2 strains to grow to 1.6 A630 units per mL (monitor in the Biotek plate reader; 5 doublings). This is ~ mid exponential phase. (remember that the linear range of the instrument is between 0.1 and 0.9- you must dilute samples in TSB to stay in this linear range). Keep detailed notes on growth rates. We are assuming for the sake of this calculation that about 4 A630 units/mL = 8×10^9 CFU/mL. Volume of saturated culture needed to obtain 8×10^{11} CFU total = $(8 \times 10^{11} \text{ CFU} / 8 \times 10^9 \text{ CFU/mL}) = 100 \text{ mL}$
- [00870] 3. Use the starter cultures from (2) to inoculate 100 mL "final" cultures of each to a density of 0.05 A630 units per mL. 1.6 mL starter is added to 98.4 mL TSB.
- [00871] 4. Allow the two strains to grow at 37C/250 rpm. Monitor the density until an A630 of 3.2 is reached. (6 doublings). Create a new culture of each strain-100 mL initiated at 0.05 A630 units/ mL (this is "round 2"). Return the flasks to the shaker 250 rpm/37C.
- [00872] 5. Harvest a 1 mL volume of cells from step 4 into 50 mL PBS for each strain.
- [00873] 5A. Snap freeze a second 1 mL of culture and place at -80C for later genetic tests (see genotypic stability below).
- [00874] 6. Centrifuge 2900 rpm for 15 min.
- [00875] 7. Aspirate the supernatant and vortex the cell pellet to resuspend.
- [00876] 8. Bring volume again to 50 mL in PBS and harvest as in step 6.
- [00877] 9. Resuspend the pellets of each strain (BioPlx-01, BioPlx-02) in pre-warmed fresh human serum 20 ml each.
- [00878] 10. Shake at 250 rpm/37C, monitoring growth. Expected outcome is that BioPlx-01 grows and BioPlx-02 (KS) does not. Collect enough data-points that the slopes of each can be calculated from semilog plots and ratioed. This ratio will be a measure of KS performance. Kill ratio (KR)=slope of BioPlx-01 growth in serum/slope of BioPlx-02 growth in serum. This KR is a measure of KS performance at 11 total doublings was reached in TSB.
- [00879] 11. The "round 2" culture from step 4A will be monitored until an A630 of 3.2 is reached (6 doublings). Use this to seed a "round 3" culture to 0.05 A630/mL,

then follow steps 5-10 using the Round 2 saturated culture. The KR is a measure of KS performance at 17 total doublings.

[00880] 12. The "round 3" culture from step 11 will be monitored until an A630 of 3.2 is reached (6 doublings), then split back again to 0.05 A630/mL. This process of growth to 3.2 followed by splitting to 0.05 was performed 4 times as follows: Round 3: was 23 doublings; Round 4: 29 doublings; Round 5: 35 doublings; Round 6: 41 doublings. Follow steps 5-10. The KR is a measure of KS performance at 41 doublings.

[00881] Plot KR as a function of culture doubling #.

[00882] Genotypic Stability:

[00883] 1. Find the samples of BioPlx-02 cells from each time point 11, 17 and 41 doublings, see step 5A.

[00884] 2. Conduct NextGen sequencing to determine the sequence homogeneity of this sample. Single molecule sequencing may be used to determine the % of mutations occurring in a population of cells at a given time point.

[00885] **Example 15. Candidate Serum and Blood Responsive Promoters screened by fluorescence to detect up-regulation**

[00886] Overview. In this example, potential *Staphylococcus aureus* promoters were tested for activity in blood and/or serum. Candidate promoters were selected from the literature based on the upregulation of gene expression after exposure to blood or serum. These promoters were then cloned upstream of a reporter molecule, green fluorescent protein (GFP), which fluoresces when the promoter is activated. After several growth steps, *Staphylococcus aureus* cells containing this promoter-GFP cassette were exposed to blood or serum, and the activity of GFP was viewed with fluorescent microscopy. The results of this screen show several promoters with varying degrees of activity in blood and/or serum, which may be used to regulate a molecular modification such as a kill switch, virulence block or nanofactory.

[00887] In example 1, a non-pathogenic strain of *Staphylococcus aureus*, denoted 502a, was used to exclude methicillin-resistant *Staphylococcus aureus* (MRSA) from the human skin microbiome. While the application of 502a has shown no adverse side effects in this trial, a kill switch was designed as an additional measure of safety. The kill switch molecular modification disclosed herein may be incorporated to target microorganisms such as *Staphylococcus aureus* 502a or RN4220 cells, and will function to inhibit cell growth, either by slowing cell growth, or promoting cell death, upon exposure to blood or serum. As such, the possibility of systemic infection in patients will be reduced or

eliminated. The kill switch comprises two key elements a kill gene to slow or stop cell growth, and a blood or serum responsive promoter to control the kill gene expression. In this example, candidate *Staphylococcus aureus* promoters were tested for increased activity in blood or serum. Candidate promoter sequences derived from *Staphylococcus aureus* strain 502a genome (NCBI CP007454.1), including about 300 bp upstream and including start codon are shown in Table 20.

[00888] Table 20. Candidate Promoter Sequences

Gene/ Description	Nucleotide sequence
<i>leuA</i>	Attttagacaattctaactattaaagtgatataaccattcacggaaggagtataataaaatgcttaataat actgaacatcaaccgacaacttcaaatattattttattatactcttaggactcgaacgtagtaaatatttact aaacgctttaagtcctatttctgttggaatgggactgtaaacgtccaataatattgggacgtttttatgtttat cttcaattactattttattactataaaacatgattaatcattaaaattacgggggaatttactatg (SEQ ID NO: 132)
<i>hlgA2</i>	Acttcaaatttcacaaactattgcgaatccattccttccactacaagcaccataattaaacaacaattca atagaataagacttgcaaaacatagttatgtcgctatataaacgcctgcgaccaataaatcttttaacataac ataatgcaaaaacatcatttaacaatgctaaaaatgtctcttcaatacatgttgatagtaattaacttttaacgaa cagttaattcgaaaacgcttacaatggattattatatatatgaactaaaattaaatagaaagaaagtgtttct atg (SEQ ID NO: 133)
<i>hrtAB</i>	Gttcatattgagttcatattcaaccttatactgacgctaaagaagaataggagagaagtgaatcgatatg (SEQ ID NO: 134)
<i>hly</i>	Ttcaggctatcaataatgcttgaaatcagcctgtagagtcataatataccaattattacatcgacgcatta agacac (SEQ ID NO: 135)
<i>sbnC</i>	Actcattgttcttattactagcaaaagggtgtatctatacattacatttctaaaagattaggtcataaaaatag caat (SEQ ID NO: 136)
<i>isdI</i>	Aactacatccgtgtattcgcatgtttagaagaaaaattaatgaagaggacaaaaaacaactaaaatttta gaaagta (SEQ ID NO: 137)
<i>isdG</i>	Tgtaatttagggaccattagggactccaaaccaataaatactgtgttacaagggttctatg (SEQ ID NO: 138)
<i>sbnE</i>	Gaatacttcaaggattaacatatagtgcattgattcaaaagtgtcatgtttgtgtcgtgaatgcgtgtcatcaac aactaaaggcacattgttggaacgacgaacagtagtttagttgttggtcaaattattggcagtccttagtggc gctgccattacaagttatactacaccagctactacgtttatcggtatgggcgtagtattgcagtaagtagttat

	ttttaattgttcaaccatcactaatcaaatcaacgatcacacattaatgaaattatgggagtgaaacaaaaaa gtg (SEQ ID NO: 139)
lrgA	Atgaaaaacgattgaatcccacttattttatactgattcatcggtcatatattattaacacgaaacacattaag aagtgcaacaatggtttaactacacttatatggtaatatgacaaatgggtgcaagatgcaagttggacgttca tttatgaaagattttaaagcgtcgataggattactttaacagtaatccttttttatgcattttacatgatattttg tatttcggactaaaaatcacgcaaatcgaagtgagccatctatacttttagttaaatcaaacgtaggaggcaat g (SEQ ID NO: 140)
lrgB	Gtttagtattattttgtattattatgtactgggtgctgtaagttaggcgaaagtcgaaaaagtaggaacgacac taacaataacattggcttactcttcgtaccagccggtatctcagttgtaactctttaggtgcttagccaag caccatttttaacattggactaataatcgtctcaacaatactattactattttgtactggctatgtcacacaaatta ttatgaaagtacttcgagatctaaaggtgacaaagtcacaaaaagatcaaaatagaggaggcacaagct catg (SEQ ID NO: 141)
hlgB	Aagatcctagagatttttcgtccagacagtgaagtacctcctctgtacaaagtggaattaaccttcatttat cgccacagtatctcatgaaaaaggttcaagcgatacaagcgaatttgaaattacttacggaagaaacatgg atgtcactcatgccattaaaagatcaacgcattatggcaacagttatttagacggacatagagtcataatgc attcgtaaatagaaactatactgttaaatcagaggtcaattggaagactcatgaaatcaaggtgaaaggaca gaattgatatg (SEQ ID NO: 142)
fhuB	Tcaaatgtaacaatgatcagaggcatatgtttaattattgctatgattctagcaggtattgcagttgctatcgc tggacaagttgcattttaggttgatgggtacccatagcaagatttttaattggaactgattatgctaaaatt ctaccattaacagccttgttaggtgggatactcgtgcttggtgccgatgtgatgcacgatatttaggagaag cgctgttggtgcaatcatttcattatcggtgttccttacttttatatttagttaaaaaaggaggacgctcaata tg (SEQ ID NO: 143)
splF	Gttcacctatattaaatagtaagcgagaagcaattgggtgttatgtatgctagtataaacaacaggtgaaa gtacaaggtcatttgcgtgtttattctcctgaaattaagaaatttattgcagataatttagataaataatcatcc atccatacattgataaatgatttttagaaattaacaacaaatcaacaattttaacatctctgtgattctatttatt cgaaatgatttaaaaaataaaactcaaaaacctaaccttatattatacgaatacttagaggagcacaaaaat g (SEQ ID NO: 144)
SAUS A300_ 2268	Gatgatgtatgttcgaatttatcaattaacatgtgaggacctccgaggaatacatggcattaaatacacgtt taatatttataaaggtgacttaattttgtcaagttgattttaccacgcttttttctttactactaagacttttgaatg aagtttaaaataattgtttatcagtgataaaatatttgaataagaagagaatggctaaataatcttaatttcag aaaagtaattgtaaccttactggcttatggtaaatattttcaatattatcgacgaggatgtgttaacaatg (SEQ ID NO: 145)

SAUS A300_ 2616	Ctatcattataatgagataatgtcatttttaattgagctaaacagacagggaaagacgattattatgattacgc atgatatgcattattgtctgagtatagttcaagaacagttgtattatcaaaggacaagtcgttgctgatacca cgccagtattgatattaaatgataaaaaaatctgtgagattgcatcattgagacaaacatcgctatttgaaatg gccgaatatatagggttagcgagccacagaaattagtacaattattattaacctgataggaagggtgaga cgccaatg (SEQ ID NO: 146)
SAUS A300_ 2617	Caggcctattttctaggaaatcgatgatttttaatatcggtcaaattattgcgaatatttttctgggcactt attgcaccaacattagatattttgattataacgaaccggctaacaagggttatacacaagggttattctctgca gtattaaatattttcagttgggtattattgggacaatattattaaaagcatatgcttcatctcaaataaaaaagg tagtttacgtaaagaataatcattttgtgaatcagatatgtaaatgaatgtagaaaggtaatgatatacatg (SEQ ID NO: 147)
isdA	CTATCTGCGGCATTTGCAGAATTACTGAATGTCGCGATGATGAT AATTAACGCTAAAATCGTTGTATTA AAAACTTTTAAAATATTTTT CAAAACATAATCCTCCTTTTTATGATTGCTTTTAAAGTCTTTAGTA AAATCATAAATAATAATGATTATCATTGTCAATATTTATTTTATA ATCAATTTATTATTGTTATACGGAAATAGATGTGCTAGTATAATT GATAACCATTATCAATTGCAATGGTTAATCATCTCATATAACAA CACATAATTTGTATCCTTAGGAGGAAAACAACATG (SEQ ID NO: 148)
isdB	CTTCAGTTGATAACTTTATTAGCACAGTTGCCTTCGCAACACTTG CCCTTTTAGGTTCAATTATCTTTATTACTTTTCAAAGAAAAGAAT CTAAATAAATCATCGTCACACTCATAACTTAATATATTTTTTTATT TTAAATTTTATTTAACCTATGTCATAGATATTTTATAATCTATAA CATAGGTTATTTTTTTATAAAATAATGTTGCAATTA ACTACCATT TCAATGTACAATACAAGTAATCAATTGATAATGATTATCAGTTG ATAATATACAATTAGGAGTTGTTTCTACAACATG (SEQ ID NO: 149)
fhuA/C	Ctttctgcagatgaataaataatgggtatgagcacacatacttaataagaagtcacggacaagttttgaa ctatgaagacttatctgtggcggtttttattttataaaagtaataatacaagacatgacaaatcgagctatccaat ttaaaaagtaatgtagtcaataagattgaaaaatgttataatgatgttcagataatcattatcaattgggatgc ctttgaaaattgataatttaaaaatagaaattttttataaacagaaagaattttattgaaagtaggggaaattat g (SEQ ID NO: 150)
ear	Tgacacctgctaattcaaacattatttgagacattctttcaattaattataaattttacctatagactagttga tatttatctacatctcaaaattctcatcaacaatcttcacatccaacattttacttttagttttataattcaaaaca

	caaaacgatgttaaaaaattattctatttttagttaatagatagttaatacattttgatatttagttaattgttctttta aaaaaatattattatattttcattgtaaacgtttacaataaaaaaaggagcaattaaaatg (SEQ ID NO: 151)
fnb	Tgtacaggcgataattatgaaacacttagtatattgttttaaattagataatgatgaatttaattgaaaaataag tataaaaaataacaagccttggtgacaagggttatgatgactgaatacaatttataggtatattcaataata aaattatcaattaacataaaaattaatgacaatcttaactttcattaactcgctttttgtattgcttttaaaaccga acaatatagactgcatttattaagttaaaaaaattaatgaattttgcatttaaggagatattatagtg (SEQ ID NO: 152)
splD	Attttaattttgatgcatacattgaacccgggaattcaggatcaccagttctaattctaacaatgaggtcat aggtgtggtgtatggcgtattggaaaaattggttctgaatataatggtgccgtatactttacgcctcaatca aagattttattcaaaagcacattgaacaataaacaatttaatatataccatgagcatgtgttcaataatttaa tgaaaaacatcggtcgaatataacataaaaaaacgtctatatcaaaagcatcatgaataaacagaggagca caaaaatg (SEQ ID NO: 153)
dps	Ataatagaaatagaatgtggaaaacaacatggcaccaaccaaatgattatgaaaaatcggtcttttagatga taatgcgaaagtaaaacttactgattgataaaacatacttgctaattgataatggatatactagatgatgaatta aaatttagacatttaaaaagcggaacaccttacatttagattagaataattataaaaaagagagtaaaaacact ttacagattagaatcattataatataataattaataaacaagcaagacgtagacaattttaaggagtgtatta aatatg (SEQ ID NO: 154)
CH52_ 00360	GAATTCTTTATAGCGCGTGCAATCACACCACAAGATAAAAGATT AAAAAGTGACAAAGCATTTATTGCATTTTATAGAAGAAACCTTCG ATCAGTTCTTACCATTTTATTCTGCATAAATAACTTTGTTTAAAT AATAGAGCACGTAATCACATCCATGATTTCGTGCTCTTTTTTCTT AATATTAAATCGAACGTTCAACATAATAATTCATACTTTTAAAA AAATTAAATAAATTTAGGTTGACCTAAACATTTTATTAGGTTA TTATATTGTCCATAAGAAGTAGAGGTGAGTCAAA (SEQ ID NO: 155)
CH52_ 00305	CATAATCCCCCTCCTTAAATTTGTTTCATATAAGATTATGATATCT TAGATTGCATAAAAAGACTAGGTTTAATAAAAATTAATAATGTGAC AAATTAACGACAAGAGAAAAATGTCAATTTTGTGACACAAATAA CATTTAATTTATTGCTATAATGTATATGTTAGAAAATTTTAATAA GTAGAATCATGCATCTAAAAGAGATTAATATTTAAGCTTCAAAT TTGAGTAAACGTGGATTACATAATTATCCCAATAAAAAAATCAT

	TACGATTAAGTTCTTTTTATGTCGTCCACATACAATAC (SEQ ID NO: 156)
CH52_01670	CATTTTATATTCCCTCCGTAAAATATAAAGTTTTCTTAAGTAGTT TATAATAATTTTAATTTGTAGTCAAAAAGACTTTGTAATAATGC GTTTCAGTTAATTATAACTTACTTATACCTTAATATAAACAACCTTA AACCCTTTTTATTATTTTAAATAACTCTAAAGTACAACCTCTAATC CGCTCTCTTTAAAAATATAAATGATAATAAGTGCACATAATTTTC TCAATGGATTTTATGAATTTAAAATATGTTATCATTTCACTAGGA CATTTGTAATATGGTATGATGCTATTTATGATTTT (SEQ ID NO: 157)
srtB	CATAAAAATCCTCTTTTATTAACGACGTTTCTTCAGTCATCACTA AACCAGTTGTTGTACCGTTTTAGATTTCGATTTTCGTTGACTTTGAC AAATTAAGTAAATTAGCATTGGACCACCGACAATCATTAATAAATA GCATTGGCTGGAATTTCTAAAGGAGGCTGTATCACTCGTCCTAA TAAATCAGCCACTAACAATAGCCATGCACCAATAACTGTAGAA AACGGAATAAGTACTCTGTAATTGCCCCCAACTAGCTTTCTAAC CACATGTGGCACAATAATACCTAAAAAGGCTAGTTGT (SEQ ID NO: 158)
sbnA	CAAAAGCGCTTCCTCCTCAAATTTAAAATTCTATAATATTGTGTG TTACCTAATTGATAATGATTCTCACTATCAAGTAATTAGGATTAT ATTTTTTATGCATTTATATGTCAAATAATTATAAGTTGCATGTAA ATCATAAATATTTTATTGACTTAGGAAAAAATTTAATTCATACT AAATCGTGATAATGATTCTCATTGTCATACATCACGAAGGAGGC TAATTAGTCAATGAATAAAGTAATTAATAATGCTTGTTGTTACGC TTGCTTTCCTACTTGTTTTAGCAGGATGTAGTGGGA (SEQ ID NO: 159)
clfA	CATTTTATTCCTCTTTTTAAAAAGTCATTTTATATTAAGTATAT ACCCTTTAAAGATATATTTAATCTCTGTTAATGGAATTATACACT AAAATTGCATTATAGCAATTAATTTGTATCGATATTTTATTATCC ACAATAATACTTTACTAACAACATTTTATTTATTGCTATTTTAA GAATTACAAACGACAACGTACGATTTGATTGCAAACATTTTTTA TTATTAATATGAACTCTACCTAATGTAATCCTAGCTTTAAATCAT

	ATTTTTTCAAAGCAGATGTGTAATTTATGGTAC (SEQ ID NO: 160)
emp homolo g	CATCTGTTATTTCTCCTTTATATAGACTCAATATTATAACCAATA TAATTTCCCTGTTATATTCATAACAGCATTATATACCAGAATTT TCAGTATAATAATTAAGTGAAGTAAACGTTGTCTTAACATTTTT ATTGTTTTTCAGCTTAAAATTAATTATTGATATTGATAGTTAAGC ATAATAATTTTTTCGTAATATAAAGTGAAAAAAGTAATAGTCCA CACCTGTTTAGAATGTGGACTATACTAGATTGCATCATTGAAAT GATGACTTTGATATTATTTATTGCTAGTTTAAAAT (SEQ ID NO: 161)
rsaC	CACGCTGTGTTTTAATGAAGTAAGATGAATTGATGTTGATGCAA CCTAAAATATTGGTATCTCCAATATTTTAGGCTACACATCAACA TAACAAAGTCGAAGGCTAATAGTCCCATATCGTGCGTTAAATAT ATATTACCCTCCTATTAATATATATACCGTTCCCGATCGCACGAT ATGGTGGTATTAGAACTTCTCTTTGAACGAAAGAGAAAAGCTAG AACTTATGCAGTTTTAATTAAGTAAACATTTGTCACCTCTTA AATCAAAGAGTAAAGTT (SEQ ID NO: 162)
hlgA1	Aacaattgtattttacaacattaattaaaaataaaagcaagacattcgtgcaatcggttaccttaaattgttta caactgtcaacaataccaagggtttattaactatatttctcacaaaattagcttttagcattccaaacaaaaag gttaaattgaacggaattatggcatttttaacttaattgtaaaaaagtgataatggtaattgttaataacagtt aattataataacgtccaaaatatattattttaattaagttaataaaaattatagaagaaagtgaaacttatg (SEQ ID NO: 163)

[00889] Initially, 21 promoter candidates were selected from literature reporting gene expression changes when *Staphylococcus aureus* cells were cultured with blood or serum. The following genes are described by Malachowa N., et al. (2011). Global changes in *Staphylococcus aureus* gene expression in human blood. PLOS ONE 6:e18617. 10.1371/journal.pone.0018617: *isdA*, *isdB*, *isdG*, *isdI*, *sbnC*, *sbnE*, *fhuA*, *fhuB*, SAUSA300_2268, SAUSA300_2616, SAUSA300_2617, *hlgB*, *lrgA*, *lrgB*, *ear*, *splD*, and *splF*. The following genes are described by Palazzolo-Ballance A. M. et al. (2008). Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. J Immunol 180(1):500-509: *fnb*, *hlyB*, *hlgB*, *isdA*, *isdB*, *isdG*, *fhuA*, *fhuB*, *dps*. Finally, Stauff D. L. et al., (2007). Signaling

and DNA-binding activities of the *Staphylococcus aureus* HssR-HssS two-component system required for heme sensing. J Biol Chem Sep 7;282(36):26111-21, describes hrtAB. In order to capture all of the relevant regulatory elements of these genes, we selected 300 base pairs upstream of the start codon of each gene as the promoter region. Each promoter region was then cloned upstream of Green Fluorescent Protein (GFP) to visualize promoter activity in media, blood, and serum. The promoters were cloned in front of GFPmut2 (a GFP variant) such that when the promoter is activated, GFP is transcribed and translated into a fluorescent protein. High fluorescence correlates with high promoter activity.

[00890] Materials and Methods

[00891] Cloning. For each blood or serum-responsive gene selected from the literature, 300 base pairs of sequence immediately upstream from the start codon was selected as the promoter region. Promoters were amplified from the 502a *Staphylococcus aureus* genome and cloned in front of GFP using either Gibson assembly (GA) or restriction enzyme (RE) digest. For Gibson assembly, promoters were amplified using primers with homology to the vector backbone. In the table below, primer sequence that matches the promoter is uppercase, while primer sequence that is homologous to the vector backbone is lowercase. For restriction enzyme digest, promoters were amplified using primers with SphI or PstI restriction sites. In the table below, primer sequence containing restriction sites is bold. The vector backbone, plasmid pCN56 (BEI Resources), was amplified using PCR for Gibson assembly, or simply digested with restriction enzymes for restriction enzyme cloning. Note that the dps promoter was never successfully cloned with GFP. After multiple attempts, the dps-GFP cassette was dropped. Final plasmid cassettes for screening are: pCN56-promoter-GFP. Primers used for amplification of promoters are shown in Table 21. Primers used for amplification of vector backbone are shown in Table 22.

[00892] Table 21. Primers used for amplification of promoters

Promoter	Cloning Method	Forward Primer (BPC#:Sequence)	Reverse Primer (BPC#:Sequence)
<i>isdA</i>	RE	366:TATAT G CAT G CCTA TCTGCGGCATTTGCAG (SEQ ID NO: 164)	367:GATAC C T G CAGGTTG TTTTCTCCTAAGGATA (SEQ ID NO: 165)

<i>isdB</i>	RE	368:GATGCGCATGCCTT CAGTTGATAACTTTATT A (SEQ ID NO: 166)	369:GATGCCTGCAGGTTG TAGAAACAACCTCCTAAT (SEQ ID NO: 167)
<i>isdI</i>	RE	379:GATACGCATGCCTT ACTCGTAGCAGTTTTTT GT (SEQ ID NO: 168)	380:GATAGCTGCAGGGG CAATCACTCCTCTATTTT (SEQ ID NO: 169)
<i>isdG</i>	RE	377:GATGCGCATGCAA ACACAAGATAATTGAA TTT (SEQ ID NO: 170)	378:GATGCCTGCAGAATT ATCCTCTTTTCTGTTTAA (SEQ ID NO: 171)
<i>sbnC</i>	RE	381:GAATCGCATGCCTT TATTAAAGCTGACAAA GTCGTA (SEQ ID NO: 172)	382:GAAATCCTGCAGTGT TCAGACACCTCGCATTC (SEQ ID NO: 173)
<i>sbnE</i>	GA	305:taactgactaggcgccgcGA ATACTTCAAGGATTAA CATATAGTGCATTG (SEQ ID NO: 174)	306:ccagtgaaaagttcttcctttactc atTTTTTTGTTTCAACTCC CATAATTTCATTAATG (SEQ ID NO: 175)
<i>lrgA</i>	GA	307:taactgactaggcgccgcAT GAAAAACGATTGAATC CCACTTATTTTATACG (SEQ ID NO: 176)	308:ccagtgaaaagttcttcctttactc atTGCCTCCTACGTTTGAT TTAACTAAAG (SEQ ID NO: 177)
<i>lrgB</i>	GA	309:taactgactaggcgccgcGT TTAGTATTATTATTTGT ATTATTATGTACTGGTG CTG (SEQ ID NO: 178)	310:ccagtgaaaagttcttcctttactc atGAGCTTGTGCCTCCTCT ATTTTG (SEQ ID NO: 179)

<i>hlgB</i>	GA	311:taactgactaggcgccgcAA GATCCTAGAGATTATTT CGTTCCAG (SEQ ID NO: 180)	312:ccagtgaaaagttcttcctttactc atATCAATTCTGTCCTTTC ACCTTGATTTC (SEQ ID NO: 181)
<i>fhuA</i>	GA	313:taactgactaggcgccgcCT TTCTTGCAGATGAATAA ATAAATGGTATGAGC (SEQ ID NO: 182)	314:ccagtgaaaagttcttcctttactc atAATTTCCCTACTTTCAA TAAAATTCTTTCTG (SEQ ID NO: 183)
<i>fhuB</i>	GA	315:taactgactaggcgccgcTC AAAATGTAACAATGAT CAGAGGC (SEQ ID NO: 184)	316:ccagtgaaaagttcttcctttactc atATTGAGCGTCCTCCTTT TTTAACTAAATATAAAAA G (SEQ ID NO: 185)
<i>ear</i>	GA	317:taactgactaggcgccgcTG ACACCTGCTAATTCAA ACATTATTTG (SEQ ID NO: 186)	318:ccagtgaaaagttcttcctttactc atTTTAATTGCTCCTTTTTT TATATTGTAAACGTTTAC (SEQ ID NO: 187)
<i>fnb</i>	GA	319:taactgactaggcgccgcTG TACAGGCGATAATTAT GAAACACTTAG (SEQ ID NO: 188)	320:ccagtgaaaagttcttcctttactc atTATAATATCTCCCTTTA AATGCAAAATTCATTAAT TTTTTTAAAC (SEQ ID NO: 189)
<i>hlb</i>	GA	321:taactgactaggcgccgcTT CAGGCTATCAATAATG CTTTGAAATC (SEQ ID NO: 190)	322:ccagtgaaaagttcttcctttactc atAGAAACCTTGTAACAA CAGTATTTATTGGG (SEQ ID NO: 191)
<i>splF</i>	GA	323:taactgactaggcgccgcGT TCACCTATATTAAATAG TAAGCGAGAAGC	324:ccagtgaaaagttcttcctttactc atTTTTGTGCTCCTCTAAG TATTCGTATAAATATAAG

		(SEQ ID NO: 192)	G (SEQ ID NO: 193)
<i>splD</i>	GA	325:taactgactaggcgccgcAT TTTAAATTTTGATGCAT ACATTGAACCCGG (SEQ ID NO: 194)	326:ccagtgaaaagttcttcctttactc atTTTTGTGCTCCTCTGTTT ATTCATGATGC (SEQ ID NO: 195)
<i>dps</i>	GA	327:taactgactaggcgccgcAT AATAGAAATAGAATGT GGAAAACAACATGGC (SEQ ID NO: 196)	328:ccagtgaaaagttcttcctttactc atATTTAATACACTCCTTA AAATTGTCTACGTC (SEQ ID NO: 197)
SAUSA 300_2268	GA	329:taactgactaggcgccgcGA TGATGTATGTTTCGAAT TTATCAATTAACATGTG (SEQ ID NO: 198)	330:ccagtgaaaagttcttcctttactc atTGTTAACACATCCTCGT CGATAATATTG (SEQ ID NO: 199)
SAUSA 300_2616	GA	331:taactgactaggcgccgcCT ATCATTATAATGAGAT AATGTCATTTTAAATTG AGC (SEQ ID NO: 200)	332:ccagtgaaaagttcttcctttactc atTGGCGTCTCACCTTCCT ATC (SEQ ID NO: 201)
SAUSA 300_2617	GA	333:taactgactaggcgccgcCA GGCCTATTTTCTAGGAA ATCGATG (SEQ ID NO: 202)	334:ccagtgaaaagttcttcctttactc atGATATATCATTACCTTT CTACATTCATTACATAT C (SEQ ID NO: 203)
<i>hlgA2</i>	GA	201:cgtaactaattaattaagaagg agatatacatACTTCAAATTT TCACAACTATTGCG (SEQ ID NO: 204)	185:ccagtgaaaagttcttcctttactc atAGAAATCACTTTCTTTC TATTTAATTTTAAGTTCA TATATA (SEQ ID NO: 205)

<i>hrtAB</i>	GA	205:cgtaaactaattaatttaagaagg agatatacatGTTTCATATTGA GTTTCATATTTCAACC (SEQ ID NO: 206)	188:ccagtgaaaagttcttcctttactc atATCGATTCACTTCTCCC TATTTCTTC (SEQ ID NO: 207)
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[00893] Table 22. Primers used for amplification of vector backbone

Plasmid	Cloning Method	Forward Primer	Reverse Primer
pCN56	GA (hlgA2, hrtAB)	197:ATGAGTAAAGGA GAAGAACTTTTCACT GG (SEQ ID NO: 208)	198:ATGTATATCTCCTTCT TAAATTAATTAGTTAACG AATTCG (SEQ ID NO: 209)
pCN56	GA (all other promoters)	197:ATGAGTAAAGGA GAAGAACTTTTCACT GG (SEQ ID NO: 210)	265:gcggccgcctagtcagttACT CAAAGGCGGTAATACGG (SEQ ID NO: 211)

[00894] Blood and Serum Samples. For blood samples, 4-8 ml of human blood was drawn into heparinized tubes and frozen. For serum samples, 4-8 ml of human blood was drawn into non-heparinized tubes, rested at room temperature for 15-30 minutes until fully clotted, and centrifuged at 3,000 rpm for 15 minutes. The serum supernatant was carefully removed, transferred to a new tube, and frozen.

[00895] Construction of Cell Lines. RN4220 *Staphylococcus aureus* cells were transformed with pCN56-promoter-GFP plasmids using electroporation. Glycerol stocks of each cell line were preserved as a starting material for the following blood/serum induction assay. Final cell lines for screening are: RN4220 + pCN56-promoter-GFP.

[00896] Blood and Serum Induction. For each cell line, 1-3 ml tryptic soy broth (TSB) media with 10 µg/ml erythromycin was inoculated with a small scoop of glycerol stock. The culture was grown at 37°C overnight shaking at 240 rpm. In the morning, the optical density (OD) of the culture was measured and the culture was used to inoculate 1

ml of fresh TSB + erythromycin to an OD of 0.1. This 0.1 OD culture was grown at 37°C shaking at 240 rpm for 2-3 hours until the OD reached 1-2. The culture was then used to inoculate three separate cultures of 500 µl of freshly thawed blood, serum, or TSB, all with erythromycin, to an OD of 0.1. These three cultures were grown at 37°C shaking at 240 rpm for 1.5-2 hours. 10 µl of each culture was dropped onto a microscope slide, covered with a coverslip, and viewed with fluorescent microscopy.

Microscopy. Images were taken with an iPhone through the eyepiece of a fluorescent microscope.

[00897] Results and Conclusions. The fluorescent images of each *Staphylococcus aureus* RN4220 + pCN56-promoter-GFP cell line cultured in either media (negative control), blood, or serum were read and fluorescence level was scored as summarized in Table 23.

[00898] Table 23. Relative promoter GFP fluorescence levels in TSB, Blood or Serum

	Fluorescence Level		
Promoter	TSB Media	Blood	Serum
isdA	high	high	high
isdB	high	(no sample)	high
isdI	low	high	high
isdG	very low	high	high
sbnC	very low	medium	medium
sbnE	very low	low	low
lrgA	very low	low	low
lrgB	very low	low	none
hlgB	very low/none	medium	medium
fhuA	high	high	high
fhuB	very low	low	low
ear	high	high	high
fnb	medium	medium	medium
hly	very low/none	medium	medium

splF	very low/none	low	low
splD	very low/none	very low/none	very low/none
SAUSA 300_2268	low	high	medium
SAUSA 300_2616	very low/none	low	low
SAUSA 300_2617	very low/none	low	low
hlgA2	low	high	medium
hrtAB	very low/none	medium	medium

[00899] The promoter for the kill switch requires two essential characteristics. First, the promoter must turn on, or be upregulated, when the cells are exposed to blood or serum. This screen clearly shows a spectrum of promoter activity in the presence of blood or serum; some promoters are very active in blood or serum, and others less so. Depending on the mechanism of activity, different kill genes will likely require promoters with different levels of activity. For example, a kill gene that is extremely lethal, rather than toxic, may require a promoter with very low strength. As various kill genes are tested, it will be possible to return to this list of promoters and rationally build kill switches.

[00900] The second requirement is that the candidate promoter must have little or no activity when the cells are not exposed to blood or serum. As the primary purpose of 502a is to colonize the skin before exposure to MRSA, it is critical that the cells grow normally in their intended niche and kill switch activity not interfere with this function. The most desirable kill switch candidate promoters in this screen exhibited very low activity in TSB and medium/high activity in blood or serum including *isdG*, *sbnC*, *sbnE*, *hlgB*, *hlb*, SAUSA300_2268, *hlgA2*, and *hrtAB*. However, *isdI*, *lrgA*, *lrgB*, *fhuB*, *splF*, *dps*, SAUSA300_2616, SAUSA300_2617 may also be useful promoter candidates for further evaluation. This screen shows several candidate promoters (*isdA*, *isdB*, *fhuA*, *ear*, and *fnb*) were active before exposure to blood and serum, so these were deprioritized from the list of potential kill switch promoters.

[00901] Additional candidate promoters were selected from the literature for future screening including *lukG*, *lukH*, *chs*, *efb*, *icaB*, SAUSA300_1059,

SAUSA300_0370, aur, and SAUSA300_0169, as described in Malachowa N, 2011 and Palazzolo-Ballance AM, 2008.

[00902] Example 16. qRT PCR for Genomic Expression of Blood and Serum-Responsive Promoters

[00903] In this example, qRT PCR was performed for 20 endogenous *Staphylococcus aureus* genes found in the literature to be blood and/or serum responsive. The screen was used to help identify candidate blood and/or serum responsive promoters for use in construction of a kill switch molecular modification comprising a cell death gene. Briefly, 502a cells were grown in TSB media, blood, or serum, and RNA was extracted at various time points. In addition, several *Staphylococcus aureus* genes were tested that are predicted to be unresponsive in blood or serum. These are considered to be candidates for a second promoter to be operably linked to an antitoxin specific for the cell death gene. The results show several genes that are upregulated in blood or serum and a few that are stable in blood or serum.

[00904] Growth Procedure. A growth experiment was performed as follows. 4ml overnight culture of 502a cells was inoculated with a small scoop of competent cells. In the morning, a 125ml disposable sterile shake flask was inoculated with 50ml of overnight culture to an optical density (OD) of 0.1. Cells were grown to an OD of 2 (several hours). At OD 2, 500ul was removed for a T=0 RNA sample. 3 x 7ml of the remaining cells were transferred to triplicate 50ml conical tubes. The tubes were spun, supernatant decanted, washed with PBS, spun again, supernatant removed, and cells resuspended in 7ml TSB, serum, or blood. Tubes were placed at 37°C with shaking at 240rpm. Additional RNA samples were collected at T=1 (tubes were sampled immediately and did not shake at 37°C), T=15 and T=45 minutes after exposure to serum or blood. RNA sampling method for TSB and serum cultures consisted of 500ul transferred to a 1.5ml tube, cells spun at 13,200 rpm for 1 minute, supernatant decanted, and 100ul of RNALater added. Sampling for blood cultures was the same, except the supernatant was aspirated, and 200ul of RNALater was added. All samples were stored at -20°C until further processing (10 months of storage).

[00905] qPCR Sample Processing and Data Analysis. RNA extraction and cDNA synthesis was performed. Frozen RNA pellets stored in RNALater were washed once in PBS, extracted using Ambion RiboPure Bacteria kit and eluted in 2 x 25ul. RNA samples were DNased using Ambion Turbo DNase kit. Samples with a final concentration less than 50ng/ul were ethanol precipitated to concentrate DNA. 10ul of DNased RNA was

used in Applied Biosystems High-Capacity cDNA Reverse Transcription kit. qPCR was performed with Applied Biosystems PowerUp SYBR Green Master Mix (10ul reaction with 1ul of cDNA). Samples were probed to look for changes in gene expression over time and in different media, and normalized to housekeeping gene, *gyrB*, using the $\Delta\Delta C_t$ method. C_t (cycles to threshold) values for *gyrB* transcripts were subtracted from C_t values for gene transcripts for each RNA sample. These ΔC_t values were then normalized to the initial time point. Primers for qRT PCR screening of candidate serum and/or blood responsive genes are shown in Table 24.

[00906] Table 24. Primers for qRT PCR screening of candidate serum and/or blood responsive genes

Gene	qRT PCR Primers (BPC#-sequence)	
	Forward	Reverse
<i>gyrB</i>	BPC802- TTGGTACAGGAATCGGTGGC (SEQ ID NO: 212)	BPC803-TCCATCCACATCGGCATCAG (SEQ ID NO: 213)
<i>isdA</i>	BPC114- GCAACAGAAGCTACGAACGC (SEQ ID NO: 214)	BPC115-AGAGCCATCTTTTGTCACTTGG (SEQ ID NO: 215)
<i>isdB</i>	BPC116- GCAACAATTTTATCATTATGCC AGC (SEQ ID NO: 216)	BPC117-TGGCAACTTTTGTCACTTCA (SEQ ID NO: 217)
<i>isdI</i>	BPC764- ACCGAGGATACAGACGAAGTT (SEQ ID NO: 218)	BPC765-TGCTGTCCATCGTCATCACTT (SEQ ID NO: 219)
<i>isdG</i>	BPC120- AACCAATCCGTAAAAGCTTGC (SEQ ID NO: 220)	BPC121-AGGCTTTGATGGCATGTTTG (SEQ ID NO: 221)
<i>sbnC</i>	BPC768- AGGGAAGGGTGTCTAAGCAAC (SEQ ID NO: 222)	BPC769-TCAGTCCTTCTTCAACGCGA (SEQ ID NO: 223)
<i>sbnE</i>	BPC124- ATTCGCTTTAGCCGCAATGG (SEQ ID NO: 224)	BPC125-GCAACTTGTAGCGCATCGTC (SEQ ID NO: 225)
<i>lrgA</i>	BPC126- GATACCGGCTGGTACGAAGAG (SEQ ID NO: 226)	BPC127-TGGTGCTGTTAAGTTAGGCGA (SEQ ID NO: 227)
<i>lrgB</i>	BPC128- ACAAAGACAGGCACAACTGC (SEQ ID NO: 228)	BPC129-GGTGTAGCACCAGCCAAAGA (SEQ ID NO: 229)

<i>hlgB</i>	BPC760- TGGTTGGGGACCTTATGGAAG (SEQ ID NO: 230)	BPC761-GGCATTTGGTGTTGCGCTAT (SEQ ID NO: 231)
<i>fhuA</i>	BPC132- CACGTTGTCTTTGACCACCAC (SEQ ID NO: 232)	BPC133-TGGGCAATGGAAGTTACAGGA (SEQ ID NO: 233)
<i>fhuB</i>	BPC134- CAATACCTGCTGGAACCCCA (SEQ ID NO: 234)	BPC135-GGGTCCGCATATTGCCAAAC (SEQ ID NO: 235)
<i>ear</i>	BPC136- CCACTTGTCAGATCTGCTCCT (SEQ ID NO: 236)	BPC137- GGTTTGGTTACAGATGGACAAACA (SEQ ID NO: 237)
<i>fmb</i>	BPC772- CGCAGTGAGCGACCATACA (SEQ ID NO: 238)	BPC773-TTGGTCCTTGTGCTTGACCA (SEQ ID NO: 239)
<i>hfb</i>	BPC140- CTACGCCACCATCTTCAGCA (SEQ ID NO: 240)	BPC141-ACACCTGTACTCGGTCGTTC (SEQ ID NO: 241)
<i>splF</i>	BPC142- TGCAATTATTCAGCCTGGTAGC (SEQ ID NO: 242)	BPC143-CCTGATGGCTTATTACCGGCAT (SEQ ID NO: 243)
<i>splD</i>	BPC144- AGTGACATCTGATGCGGTTG (SEQ ID NO: 244)	BPC145-AACACCAATTGCTTCTCGCTT (SEQ ID NO: 245)
<i>dps</i>	BPC146- AGCGGTAGGAGGAAACCCTG (SEQ ID NO: 246)	BPC147-GTTCTGCAGAGTAACCTTTTCG (SEQ ID NO: 247)
<i>srtB</i>	BPC846- TGAGCGAGAACATCGACGTAA (SEQ ID NO: 248)	BPC847-CCGACATGGTGCCCGTATAA (SEQ ID NO: 249)
<i>emp</i>	BPC854- TCGCGTGAATGTAGCAACAAA (SEQ ID NO: 250)	BPC855-ACTTCTGGGCCTTTAGCAACA (SEQ ID NO: 251)
<i>sbnA</i>	BPC858- CCTGGAGGCAGCATGAAAGA (SEQ ID NO: 252)	BPC859-CATTGCCAACGCAATGCCTA (SEQ ID NO: 253)
CH52_36 0	BPC834- TTCAACTCGAACGCTGACGA (SEQ ID NO: 254)	BPC835-TTGCACCCATTGTTGCACCT (SEQ ID NO: 255)
CH52_30 5	BPC838- TTCCTGGAGCAGTACCACCA (SEQ ID NO: 256)	BPC839-CAGCGCAATCGCTGTAAACTA (SEQ ID NO: 257)
CH52167 0	BPC842- GCGATTATGGGACCAAACGG (SEQ ID NO: 258)	BPC843-ACTTCATAGCTTGGGTGTCCC (SEQ ID NO: 259)

<i>clfA</i>	BPC850- TCCAGCACAACAGGAAACGA (SEQ ID NO: 260)	BPC851-TAGCTTCACCAGTTACCGGC (SEQ ID NO: 261)
SAUSA3 00_2268	BPC778- GCTTCTACAGCTTTGCCGAT (SEQ ID NO: 262)	BPC779-GATTTGGTGCTTACTGCCACC (SEQ ID NO: 263)
SAUSA3 00_2616	BPC774- ACAAGCGCAACAAGCAAGAG (SEQ ID NO: 264)	BPC775- TGCGTTTGATACCTTTAACACGG (SEQ ID NO: 265)
SAUSA3 00_2617	BPC152- GGGCTGAAAAAGTTGGCATGA (SEQ ID NO: 266)	BPC153-ACGCGTTGTTTTTGACCTCC (SEQ ID NO: 267)
hlgA2	BPC179- TGATTTCTGCACCTTGACCGA (SEQ ID NO: 268)	BPC180-AGCCCCCTTTAGCCAATCCAT (SEQ ID NO: 269)
hrtAB	BPC713- ACACAACAACAACGTGATGAG C (SEQ ID NO: 270)	BPC714-TAACGGTGCTTGCTCTGCTT (SEQ ID NO: 271)

[00907] The qPCR results are shown in FIGs 13A and 13B showing several genes that are upregulated in blood and /or serum. FIG. 13A shows promoter candidates *isdA*, *isdB*, *hlgA2*, *hrtAB*, *isdG*, *sbnE*, *lrgA*, *lrgB*, *fhuA*, *fhuB*, *ear*, *hlb*, *splF*, *splD*, *dps*, and SAUSA300_2617 at 1 min, 15 min and 45 min in serum and fold changes in gene expression vs. media. Preferred serum responsive promoter candidates in this screen include *hlgA2*, *hrtAB*, *isdA*, *isdB*, *isdG*, *sbnE*, *ear*, and *splD*, as shown in Table 25 because they exhibit at least a 9-fold increase in gene expression when exposed to serum after 45 min, a slightly delayed response to serum, and are not significantly upregulated at T=1 min.

[00908] Table 25. Preferred promoter candidates for serum-responsive genes by qPCR

Upregulated Gene	Fold Change in Serum at T=45 min
<i>hlgA2</i>	9
<i>hrtAB</i>	209
<i>isdA</i>	15
<i>isdB</i>	172
<i>isdG</i>	42
<i>sbnE</i>	30
<i>ear</i>	10
<i>splD</i>	9

[00909] FIG. 13B shows candidate promoter activity when exposed to blood of promoter candidates *isdA*, *isdB*, *hlgA2*, *hrtAB*, *isdG*, *sbnE*, *lrgA*, *lrgB*, *fhuA*, *fhuB*, *ear*, *hlb*, *splF*, *splD*, *dps*, and SAUSA300_2617 at 1 min, 15 min and 45 min in serum and fold changes in gene expression vs. media by qPCR. Preferred promoter candidates exhibited a slightly delayed gene expression response at 1 minute, but were significantly upregulated at least 30-fold the 15 and 45 min time points. Preferred promoter candidates for blood-responsive genes by qPCR included *isdA*, *isdB*, *isdG*, *sbnE*, and SAUSA300_2617, as shown in Table 26.

[00910] Table 26. Preferred promoter candidates for blood-responsive genes by qPCR

Upregulated Gene	Fold Change in Blood at T=45
<i>isdA</i>	77
<i>isdB</i>	66
<i>isdG</i>	69
<i>sbnE</i>	33
SAUSA300_2617	150

[00911] Another qRT PCR for Genomic Expression of Serum-Responsive Promoters

In this example, qRT PCR is also performed for screening further *Staphylococcus aureus* genes found in the literature to be blood and/or serum responsive. Briefly, 502a cells were grown in TSB media or serum, and RNA was extracted at various time points. The results show several genes that are highly upregulated in serum. Essentially, the experimental protocol was similar to the example above, except RNA samples were normalized before conversion to cDNA, and samples were collected at T=90 min.

[00912] Growth Procedure. The growth experiment was performed as follows. 502a glycerol stock was struck onto a fresh bacterial plate and grown overnight. 3-5 single colonies from the plate were inoculated into a 4ml culture of BHI media and grown overnight at 37°C with shaking at 240rpm. In the morning, the culture was diluted to an optical density (OD) of 0.05 in 5ml fresh BHI media. Cells were grown at 37°C with shaking at 150rpm for several hours to an OD of approximately 1. At this time, samples for RNA were collected for a T=0 time point (1ml was transferred to a 1.5ml microcentrifuge tube, centrifuged at 16,000rpm for 1 minute, supernatant dumped, cells

resuspended in 1ml sterile PBS, centrifuged at 16,000rpm for 1 minute, supernatant aspirated, cells resuspended in 200ul RNALater, and stored at -20°C). The remaining culture was rediluted to an OD of 0.05 in 3 replicate heparinized tubes of 10ml fresh BHI media or thawed human serum, and incubated at 37°C with shaking at 150rpm. Additional samples for RNA were collected at T=90 minutes, and T=180 minutes. For these later samples, one 10ml tube was centrifuged at 3,000rpm for 10 minutes, supernatant dumped, cells resuspended in 1ml PBS, transferred to a 1.5ml microcentrifuge tube, centrifuged at 16,000rpm for 1 minute, supernatant aspirated, cells resuspended in 200ul RNALater, and stored at -20°C.

[00913] qPCR Sample Processing and Data Analysis. RNA extraction and cDNA synthesis was performed as follows. Frozen RNA pellets stored in RNALater were washed once in PBS, extracted using Ambion RiboPure Bacteria kit and eluted in 2 x 50ul. RNA samples were DNased using Ambion Turbo DNase kit. Samples with a final concentration less than 50ng/ul were ethanol precipitated to concentrate DNA. 500ng of DNased RNA was used in Applied Biosystems High-Capacity cDNA Reverse Transcription kit. qPCR was performed with Applied Biosystems PowerUp SYBR Green Master Mix (10ul reaction with 1ul of cDNA).

Samples were probed to look for changes in gene expression over time and in different media, and normalized to housekeeping genes, *gyrB*, *sigB*, *rho*, or an average of the three, using the $\Delta\Delta C_t$ method. C_t (cycles to threshold) values for housekeeping gene transcripts were subtracted from C_t values for gene transcripts for each RNA sample. These ΔC_t values were then normalized to the initial time point. Gene expression at 90 minutes in both TSB and serum were normalized to values at T=0.

[00914] Results are shown in FIG. 13C which shows gene expression in serum at T=90 min for promoter candidates *hlgB*, *ear*, *fnb*, *splF*, *splD*, *clfA*, CH52_360, CH52_305, CH52_1670, *hly*, *lrgB*, *lrgA*, *emp*, *fhuA*, *fhuB*, *isdI*, *isdA*, *srtB*, *isdG*, *sbnE*, *sbnA*, *sbnC*, and *isdB* by qPCR compared to TSB. FIG. 13C shows genes upregulated greater than 5-fold in serum include *fhuA*, *fhuB*, *isdI*, *isdA*, *srtB*, *isdG*, *sbnE*, *sbnA*, *sbnC*, and *isdB*. FIG. 13C shows several genes are upregulated greater than 100-fold after 90 minutes of incubation in serum including *isdA*, *srtB*, *isdG*, *sbnE*, *sbnA*, *sbnC*, and *isdB*. Specifically, genes in the *isd*, *sbn*, and *fhu* families are upregulated to varying degrees. All of the genes surveyed here have stable expression from T=0 to T=90 minutes in TSB. Several genes from this experiment show high upregulation in serum, while others show stable expression in serum. Both of these characteristics may be useful in construction of

a kill switch. For example, a cell death gene may be controlled with a promoter that will upregulate in serum and/or blood, and an antitoxin gene specific for the cell death gene may be controlled with a promoter that will downregulate or remain stable in serum.

[00915] Example 17. *SprA1* as a candidate cell death gene toxin using plasmid based induction systems

[00916] In this example, candidate cell death gene *sprA1* was evaluated using two different plasmid based induction systems in two *Staphylococcus aureus* strains.

Example 17A. Initial testing of *sprA1* as an inhibitor of cell growth of *Staph aureus* cells (RN4220) was performed using a cadmium inducible promoter. A *sprA1* toxin gene was cloned behind the cadmium promoter in pCN51 (pTK1). pCN51 vector is a low copy plasmid containing a cadmium inducible promoter.

[00917] This version of *sprA1* contains an antisense which regulates *sprA1*. The full sequence of the *sprA1-sprA1AS* which is downstream of the cadmium promoter is shown below. This construct is called pTK1.

[00918] pTK1: *sprA1-sprA1AS*: *sprA1* toxin gene and ribosome binding site, and antitoxin gene (pTK1 or p001). pTK1 was used in experiments with Cadmium promoter.

CGCAGAGAGGAGGTGTATAAGGTGATGCTTATTTTCGTTACATCATAGCA
CCAGTCATCAGTGGCTGTGCCATTGCGTTTTTTTCTTATTGGCTAAGTAGAC
GCAATACAAAATAGGTGACATATAGCCGCACCAATAAAAATCCCCTCACT
ACCGCAAATAGTGAGGGGATTGGTGTATAAGTAAATACTTATTTTCGTT
GT (SEQ ID NO: 272)

ribosome binding site region

sprA1 toxin gene

***sprA1* antitoxin gene**

CCCCTCACTACCGCAAATAGTGAGGGGATTGGTGTATAAGTAAATACTTAT
TTTCGTTGT (SEQ ID NO: 273)*sprA1* antitoxin gene

[00919] Cadmium is a toxic compound so the first step was to find the sub-inhibitory concentration in which the cadmium has enough of a minimal effect on growth to see a marked delta if *sprA1* is having a negative on growth of RN4220. RN4220's were grown overnight in TSB media and diluted down to 0.5 ODs and separated into eight 14 ml culture tubes each containing 3 ml of diluted RN4220 cells. Four concentrations of cadmium were inoculated into 4 tubes with each having no cadmium control. 10nM, 100nM, 1uM and 10uM were the final cadmium concentrations. The

results were evaluated at 2 and 22 hours of growth at 30 °C with 240 RPM shaking (data not shown). After 22 hours the 10uM Cadmium showed the greatest negative effect. The experiment of determining the minimal sub-inhibitory concentration of cadmium was repeated in duplicate using 10nM, 100nM and 1uM cadmium using *Staphylococcus aureus* RN4220 cells. After 2 hours, cell growth results from the cadmium test show good tolerance up to 1uM (data not shown).

[00920] Next, 500nM and 1uM cadmium was tested using RN4220 cells transformed with pCN54 which has a cadmium inducible promoter was used as an additional control. RN4220 cells were diluted to 0.5 ODs (630nm) and aliquoted to 4 culture tubes each with 3 ml. Two of the tubes were inoculated with 500nM and 1uM cadmium. RN4220 cells containing pCN54 were diluted to 0.5 ODs (630nm) and aliquoted to 4 culture tubes each with 3 ml. Two of the tubes were inoculated with 500nM and 1uM cadmium. All pCN54 growths contained erythromycin 10 as an antibiotic selection. After 2 hours of growth at 30 °C, ODs (630 nm) were measured. Results showed good tolerance at 500nM and 1uM cadmium. (data not shown). It was concluded that the 4220 cells exhibited good cadmium tolerance at the levels tested except for 10uM which was too high of a concentration to potentially see a difference between cadmium effects only and an induced toxin.

The next experiments included a toxin (sprA1) behind a cadmium promoter on a pCN51 plasmid (pTK1) which had been transformed into RN4220 cells. Both 500nM and 1uM concentrations were tested with 2 pTK1 clone picks and RN4220 cells (wt). Overnight cultures of wt RN4220 cells and two clones of pTK1 in RN4220 cells were diluted to 0.5 ODs. Wild-type (WT) RN4220 cells were divided into 3 culture tubes at 3 ml/tube. Two tubes were inoculated with 500nM and 1uM cadmium and ODs were read after 2 hours post induction. Each pTK1 clone was divided into 3 culture tubes at 3 ml/tube (6 tubes total). Each pTK1 clone was induced with 500nm and 1uM with one being a control. ODs were read after 2 hours post induction. Results are shown in the Table 27 and FIG. 14.

[00921] FIG. 14 shows inducible inhibition of cell growth of synthetic microorganism pTK1 cells comprising a cell death toxin gene (sprA1) behind a cadmium promoter on a pCN51 plasmid (pTK1) which had been transformed into *Staphylococcus aureus* RN4220 cells. OD (630 nm) read at 2 hrs post induction, as shown in Table 27. Wild-type 4220 cells showed good cell growth both in the absence of cadmium and in

the presence of 500 nM and 1 uM cadmium. pTK1-1 and pTK1-2 cells showed good growth in the absence of cadmium, but cell growth was significantly inhibited in presence of 500 nM and 1 uM cadmium at 2 hours post induction.

[00922] Table 27. *Staphylococcus aureus* RN4220 cells Optical Density (630 nm) 2 hours post-induction

Cells	2 Hr Post OD (630 nm)
WT4220 Cad -	3.0
WT 4220 Cad+ 500nM	2.9
WT 4220 Cad+ 1uM	2.9
ptK1-1 Cad-	2.6
pTK1-1 Cad+ 500nM	0.19
pTK1-1 Cad+ 1uM	0.25
ptK1-2 Cad-	2.4
pTK1-2 Cad+ 500nM	0.16
pTK1-2 Cad+ 1uM	0.22

[00923] The experiment was reproduced and each sample exhibited similar OD (630 nm) results at 2 hrs post-induction (data not shown). In summary, a cadmium tolerance test was performed on wt RN4220 cells and 500nM-1uM cadmium showed minimal negative on RN4220 cells. This example shows induction of pTK1 showed suppression of cell growth when induced with cadmium.

[00924] Example 17B. Candidate cell death gene SprA1 was evaluated as an inhibitor of cell growth of Staph aureus cells (502a) using an anhydrotetracycline (ATc) inducible promoter: pRAB11 which is a high copy plasmid containing a tetracycline inducible promoter. Two versions of the sprA1 toxin were cloned behind the tet promoter in pRAB11-2. Clones tested were p174 plasmid containing a deleted sprA1 antisense (Das) and p175 plasmid which contains a deleted sprA1 antisense plus a missing RBS site. A plasmid map of p174 (pRAB11_Ptet-sprA1) is shown in FIG. 15A and 15B. FIG. 15A shows a zoomed view of the region of the plasmid containing the Ptet-sprA cassette. FIG. 15B shows the p174 whole plasmid in its native circular form.

[00925] Sequences employed in p174 and p175 are shown below. Both p174 and p175 were used in experiments using a tetracycline promoter

[00926] p174 sprA1: sprA1 toxin gene and ribosome binding site (p174):
 CGCAGAGAGGAGGTGTATAAGGTGATGCTTATTTTCGTTACATCATAGCA
 CCAGTCATCAGTGGCTGTGCCATTGCGTTTTTTTCTTATTGGCTAAGTAGAC
 GCAATACAAAATAGGTGACATATAGCCGCACCAATAAAAAT (SEQ ID NO:
 274)

[00927] p175 sprA1(ATG): sprA1 toxin gene beginning at start codon (ribosome
 binding site removed) (p175):
 ATGCTTATTTTCGTTACATCATAGCACCAGTCATCAGTGGCTGTGCCATTG
 CGTTTTTTTCTTATTGGCTAAGTAGACGCAATACAAAATAGGTGACATATAG
 CCGCACCAATAAAAAT (SEQ ID NO: 275)

[00928] Cell growth. Specifically, tet inducible genes on the pRAB11 vector in
 502a cells were grown overnight growths in BHI. The p174 pRAB11-pro-tet-sprA1Das
 exhibited 5.4 OD. The p175 pRAB11-pro-tet-sprA1Das(ATG) exhibited 6.2 OD. All 5
 overnight cultures were diluted to 0.5 ODs in 1 ml final (14ml tubes) of BHI-chlor10
 (502a wt just BHI). Each cell line was divided into 2 tubes for non-induced and induced
 anhydrotetracycline (ATc)-10 total.

Induction. Literature shows induction at 100 ng/ml of ATc is effective, so this
 concentration was selected for induction in these experiments. One tube from each set
 was induced with 100 ng/ml final concentration. A 1 mg/ml ATc stock in Ethanol was
 diluted to 100 ug/ml in EtOH. One microliter was added to the appropriate tubes for a
 final of 100 ng/ml.

[00929] The OD's at 630nm were taken at 2, 4 and 6 hours. The ODs were at 2
 and 4 hours were read at a 1/10 dilution while the 6 hour OD was taken at a 1/100 dilution
 to make sure readings were staying in the linear range.

[00930] The 502a's (non-induced and induced) and p174 (pRAB11-pro-tet-
 sprA1Das) tubes were serially diluted to 10e-5 and 10e-6 for dilution plating onto BHI
 and BHI-chlor10 respectively.

[00931] Results are shown in Tables 28 and 29 for ODs, and a plate comparison
 picture is shown in FIG. 15C.

Table 28. Calculations Table for Induction growth curves.

Sample Name	O/N OD	ul O/N culture	BHI	# of tubes	conditions
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502a wt	4.7	106	1 ml	2	Un-ind.& Induced
502a p174 pRAB11-ptet-sp a1Das	5.4	93	1 ml	2	Un-ind.& Induced
502a p175 pRAB11-ptet- spa1Das(ATG)	6.2	81	1 ml	2	Un-ind.& Induced

[00932] Table 29. 502a pRAB11 tet induction experiment

	OD ₆₃₀ readings at time point (hours)			
Sample Name	0.0	2.0	4.0	6.0
502a wt	0.5	4.8	8.0	14
502a wt + 100 ng ATc	0.5	4.4	7.1	11
502a p174 pRAB11-ptet- spa1Das	0.5	4.5	7.7	13
502a p174 pRAB11-ptet- spa1Das + 100 ng ATc	0.5	0.7	0.3	0
502a p175 pRAB11-ptet- spa1Das(ATG)	0.5	4.3	7.7	7
502a p175 pRAB11-ptet- spa1Das(ATG) + 100ng ATc	0.5	3.8	8.1	13

[00933] FIG. 15C shows plate dilutions at 10e-5 after 6 hours of induction for uninduced (left) and induced (right) 502a p174 (tet-spra1Das). The Plate on the left = uninduced p174 (tet-spra1Das) at 10e-5 dilution on BHI chlor10. Plate on the right is the induced p174 (tet-spra1Das) at 10e-5 on BHI chlor10. Both plates are samples from post-

induction time point of 6 hrs. The plate on the left (Uninduced) was uncountable at 10e-5 but at 10e-6 counted ~ 720 colonies. The induced plate on the right at 10e-5 produced 16 colonies as shown in Table 30.

[00934] Table 30. Survival percentage of induced *Staphylococcus aureus* 502a p174 (tet-spr1Das) cells at 6 hours post-induction

Condition	Colonies	Countable Dilution	Calculation for 0.1 mls plated	CFU's/ml
Uninduced	720	10e-6	$(720 \times 10e6)/0.1$	$7.2 \times 10e9$
Induced	16	10e-5	$(16 \times 10e5)/0.1$	$1.6 \times 10e7$

As shown in Table 30, the survival percentage of induced cells at 6 hours post-induction was calculated as $1.6 \times 10e7 / 7.2 \times 10e9 = .00222 \times 100 = 0.222\%$. The survival percentage of induced *Staphylococcus aureus* 502a p174 (tet-spr1Das) cells at 6 hours post-induction was only 0.222% compared to uninduced cells. Therefore, the *Staphylococcus aureus* 502a p174 cells exhibited $100\% - 0.222\% = 99.78\%$ measurable average cell death at 6 hours post-induction compared to uninduced cells.

[00935] In summary, induction with 100ng/ml ATc showed good suppression of growth of p174 in 502a cells up to 6 hours post induction of less than 1%, less than 0.5%, or less than 0.25%. Specifically, CFU counts at the end of 6 hours showed a survival percentage of only 0.22% when compared to the uninduced sample and 502a wild type. Induction of p175 control with the deleted RBS site for spr1 showed no negative effects on growth up to 6 hours. In summary, induction of p174 showed suppression of cell growth when induced with ATc. However, induction of p175 control lacking RBS showed no suppression of cell growth when induced with ATc, comparable to 502a wild type cells.

[00936] Example 18. 502a Inducible Plasmid Based Expression of various Toxin Genes

[00937] This example shows the effectiveness of various candidate cell death toxin genes that may be used for a kill switch in *Staphylococcus aureus* 502a. A plasmid based inducible toxin expression was used for this experiment. pRAB11 is a high copy plasmid in *Staph aureus Staphylococcus aureus*, and the Ptet promoter is derepressed by the addition of 100 ng/mL of AtC (anhydrotetracycline), allowing for high transcription

rates. pRAB11 is described in Helle, Leonie, et al. "Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*." *Microbiology* 157.12 (2011): 3314-3323. Four candidate cell death toxin genes were selected for evaluation: sprA1, 187-lysK, Holin, and sprG.

[00938] sprA1(PepA1). The gene srpA1 found in *Staphylococcus aureus* strains has been shown to code for a small membrane toxin PepA1. Sayed, Nour et al. "Functional and Structural Insights of A *Staphylococcus Aureus* Apoptotic-like Membrane Peptide from a Toxin-Antitoxin Module." *Journal of Biological Chemistry*, vol. 287, no. 52, 2012, pp. 43454–43463, doi:10.1074/jbc.m112.402693. Sayed et al. described how the sprA1 gene codes for the toxin protein called PepA1, which localizes at the bacterial membrane and causes cell death. This is part of a type I toxin antitoxin system in *Staphylococcus aureus*, and has been evolutionarily preserved in their genome.

[00939] 187-lysK. This is an engineered phage lysin protein from the *Staphylococcus aureus* phage K. Horgan, Marianne, et al. "Phage lysin LysK can be truncated to its CHAP domain and retain lytic activity against live antibiotic-resistant staphylococci." *Applied and environmental microbiology* 75.3 (2009): 872-874. O'Flaherty et al. designed and truncated this peptide and determined it to still retain is lytic activity for many *Staphylococcus aureus* strains. O'Flaherty, S., et al. "The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant staphylococci, including methicillin-resistant *Staphylococcus aureus*." *Journal of bacteriology* 187.20 (2005): 7161-7164.

[00940] holin. The holin toxin we tested in this experiment is part of the genome of many lytic phages that target *Staphylococcus aureus*. It has been shown to disrupt cell growth in *E. coli* when induced from a plasmid expression vector by forming lesions in the cellular membrane. Song, Jun, et al. *Journal of General Virology* 97.5 (2016): 1272-1281.

[00941] sprG. The coding region termed sprG is part of another type I toxin antitoxin system in *Staphylococcus aureus*. Two peptides are coded for in the same reading frame of sprG, and both have been shown to cause cell death when induced. Pinel-Marie et al. *Cell reports* 7.2 (2014): 424-435.

[00942] Materials. Various synthetic strains were prepared as shown below and 502a wt was also employed. Strains include:

- BP_068 (502a pRAB11-Ptet-sprA1)
- BP_069 (502a pRAB11-Ptet-187lysK)

- BP_070 (502a pRAB11-Ptet-holin)
- BP_071 (502a pRAB11-Ptet-sprG1)
- BP_001 (502a wt).

[00943] Growth Media used in this example included BHI broth media (37 g/L) (Alpha Biosciences), BHI agar plates, BHI Chloramphenicol (10µg/mL(Teknova)) agar plates, and BHI Chlor (10µg/mL (Teknova)) + AtC (100 ng/mL (Alfa Aesar)) agar plates. Table 31 below shows a list of oligonucleotide sequences used for constructing the plamids.

[00944] Table 31. List of oligos and their sequences used for constructing plasmids

Oligo Name	DNA sequence (5' - 3')
BPC_670	GCTCAGATCTGTTAACGGTACCATCATACTC (SEQ ID NO: 276)
BPC_671	CACTGGCCGTCGTTTTACAAC (SEQ ID NO: 277)
BPC_672	gagtatgatggtaccggttaacagatctgagcCGCAGAGAGGAGGTGTATA AGGTG (SEQ ID NO:278)
BPC_674	gagtatgatggtaccggttaacagatctgagcATGGTGGCATTACTGAAATC TTTAGAAAG (SEQ ID NO: 279)
BPC_675	gagtatgatggtaccggttaacagatctgagcATGGCACTGCCTAAAACGG G (SEQ ID NO:280)
BPC_676	gagtatgatggtaccggttaacagatctgagcATGGCTAATGAACTAAAC AACCTAAAGTT (SEQ ID NO: 281)
BPC_677	gttgtaaaacgacggccagtGCCGGGCTCAGCTATTATCA (SEQ ID NO:282)
BPC_678	gttgtaaaacgacggccagtGCGGCCGCCCATGCATGC (SEQ ID NO:283)

[00945] Table 32 shows the DNA sequence and amino acid sequence for toxin genes. *sprA1*, 187-lysK, *holin*, and *sprG* were tested in this experiment. The toxin gene *sprG* has two reading frames which have both been shown to have toxin activity in *Staphylococcus aureus*. The shorter sequence is in bold.

[00946] Table 32. DNA and amino acid sequences for toxins

Toxin	DNA Sequence	Protein Sequence
<i>sprA1</i>	ATGCTTATTTTCGTTACATCATA GCACCAGTCATCAGTGGCTGTGC CATTGCGTTTTTTTCTTATTGGCT AAGTAGACGCAATACAAAATAG (SEQ ID NO: 284)	LIFVHIIAPVISGCAIAFFSYWL SRRNTK (SEQ ID NO: 285)
187-lysK	Atggcactgcctaaaacgggtaaaccaacggcaaa acaggtggttgactgggcaatcaatataatcggcagtg gtgtcgatggtgatggtattatggtcggcaatggtggg atttacctaactatattttaatagatactggaactttaag acaccaggcaacgcaagagatatggcatggtataga tatcctgaagggttaaagtgttagaaacacttctgatt ttgtccctaaaccaggtgatatacagtggtggacaggt ggtaattacaattggaacacttggggacacactggtat tggttaggtccatcaactaaaagtactttatagtga gatcagaattggaataactctaactcttacgttggtagt cctgcagcaagataaaacatagttattttggtgtaact cattttgtagaccgcatacaaaagcagaaccgaaac ctacaccactggacagtacaccggcaactagac cagttacaggttcttgaaaaagaaccagtacggaac ttggtataaacggaaaatgcaacattgtcaatggtga accaacctatagtaactagaataggttctccattcttaa atgctccagtaggcggtaacttaccggcaggggcta caattgtatatgacgaagttgtatccaagcaggtcac atttgataggttataatgcttacaacggtaacagagta tattgccctgtagaactgtcaaggtgtccacctaate aaatacctggcggtgctggggagtattcaaa (SEQ ID NO: 286)	MALPKTGKPTAKQVVDWAIN LIGSGVDVDGYGRQCWDLPL NYIFNRYWNFKTPGNARDMA WYRYPEGFKVFRNTSDFVPK PGDIAVWTGGNYNWNTWGH TGIVVGPSTKSYFYSDQNW NNSNSYVGSPAARIKHSHYFGV THFVRPAYKAEPKPTPLDST PATRPVTGSWKKNQYGTWY KPENATFVNGNQPIVTRIGSPF LNAPVGGNLPAGATIVYDEV CIQAGHIWIGYNAYNGNRVY CPVRTCQGVPPNQIPGVAWG VFK (SEQ ID NO: 287)
Holin	Atggctaataaactaaacaacctaagttgttgag gaataaactttagcacaagaactaagagtaaaacattt tggttagcaattatcagcagtagcagttattgctaata caaattacaggtgcttttggttagactactcagctcaa attgagcaaggtgtaatatcataggttctataactaaca ttattagcaggttaggtattattgtgataataataactaa aggtcttaagatagtgatattgttcaaacagattatata aaacctcgtgatagtaaagaccctaataatgaattgtcaa tggaagcaaatgcaaacacagctagcactttcgat tagacaactatgaaaacaatgcagaacctgatacaga	MANETKQPKVVGGINFSTR KSKTFWVAIISAVVFANQIT GAFGLDYSAQIEQGVNIIGSIL TLLAGLGIIVDNNTKGLKDS IVQTDYIKPRDSKDPNEFVQW QANANTASTFELDNYENNAE PDTDDSDEVP AIEDEIDGGS PSQDEEDTEEHGKVF AEEV (SEQ ID NO: 289)

	tgatagtgatgaagtacctgctattgaagatgaaattga tggcgggtcagcaccttctcaagatgaagaagatacc gaggaacacggtaaagtatttcagaggaggaagtt aagtag (SEQ ID NO: 288)	
<i>sprG</i>	ATGGTGGCATTACTGAAATCTTT AGAAAGGAGACGCCTAATGATT ACAATTAGTACCATGTTGCAGT TTGGTTTATTTCCTTATTGCATT GATAGGTCTAGTAATCAAGCTT ATTGAATTAAGCAATAAAAAAT AA (SEQ ID NO: 290)	MVALLKSLERRRLMITISTM LQFGLFLIALIGLVIKLIELS NKK (SEQ ID NO: 291)
<i>sprA2</i>	ATGTTCAATTTATTAATTAACAT CATGACTTCAGCTTTAAGCGGCT GTCTTGTTGCGTTTTTGCACATT GGTTACGAACGCGCAACAATAA AAAAGGTGACAAATAA (SEQ ID NO: 304)	MFNLLINIMTSALSGCLVAFF AHWLRTRNNKKGDK (SEQ ID NO: 305)

[00947] Methods

Plasmid Construction was performed as follows.

- 1) PCR amplify pRAB11 backbone using primers BPC_670 and BPC_671 using an empty vector as a template.
- 2) PCR amplify toxin genes from synthesized plasmid DNA (Genscript). This allows for designing a primer that binds to the plasmid backbone downstream of the target gene, negating the need to design and order unique primers for both ends of each gene.
- 3) Primer pairs
 - a) *sprA* - BPC_672/BPC_677
 - b) 187-lysK - BPC_675/BPC_678
 - c) Holin - BPC_676/BPC_678
 - d) *sprG* - BPC_674/BPC_678
- 4) Run PCR products to check for correct size, digest the template DNA with DpnI (NEB), and clean up the reactions with a Zymo spin column.
- 5) Assemble the cleaned up PCR products by Gibson Assembly and transform into electrocompetent IM08B *E. coli* cells using the manufacturers protocol (NEB).
- 6) Verify correct sequences for the promoter and toxins on the plasmids.

- 7) Transform sequence verified plasmids into electrocompetent *Staphylococcus aureus*.

[00948] Growth Experiments were performed as follows.

- 1) Start overnight cultures of each strain in 5mL BHI broth media. Add 10ug/mL Chloramphenicol to the media for strains BP_068-BP_071.
- 2) Perform a 1:100 dilution of the overnight culture into fresh BHI. Add 10ug/mL chloramphenicol to the media for strains BP_068-BP_071. Incubate at 37°C shaking at 250rpm for 2 hours. Streak a plate of each strain and incubate overnight at 37°C to confirm cultures are good.
- 3) Take OD600 readings of 2hr cultures and dilute the cultures to an OD of 0.05
 - a) Each strain gets (4) 5 mL tubes with BHI broth
 - b) The following table shows the recorded OD readings, and the calculated amounts of each culture used to inoculate fresh cultures to an OD of 0.05.

Table 33

[00949] Table 33. Starting OD₆₀₀ readings.

Strain	OD600	uL inoculum	Calculated starting OD
BP_068	2.1	119	0.0499
BP_069	1.7	147	0.0498
BP_070	2.0	125	0.05
BP_071	1.8	139	0.05
BP_001	1.7	147	0.0498

- 4) Save 100uL sample of each culture for dilution plating. (3 plates/culture)
- 5) Incubate cultures at 37°C until the OD reaches 0.5. Add 150 ng/mL anhydrotetracycline (AtC) to 2 tubes for each strain and label them with a + to indicate they received the inducer (derepressor). Continue to grow the cells for another 4 hours taking samples as described below.
- 6) Take OD600 readings at T=30 min, 60 min, 120 min, and 240 min. Record values in the table below

- a) Perform dilution plating at T= 0, 60 min, and 240 min, and plate the correct dilution on the following plates (BHI, BHI Chlor10, BHI Chlor10+AtC 0.1)

[00950] Cfu investigation was performed as follows.

- 1) Identify BHI (Chlor 10, AtC 0.1) agar plates with colonies growing on them from strains containing plasmids with toxin genes present. Plates from T240 would be best.
- 2) If possible, pick 8 colonies per strain. Patch colonies to new BHI (Chlor 10, AtC 1) agar plate, and perform *Staphylococcus aureus* lysis procedure. Use 5uL of the lysis reaction as the template for colony PCR using primer DR_215/DR_216 using a HF polymerase, such as Q5/Phusion.
 - a) Reactions that produce a good band, perform DpnI digest for 1 hr, and column purify PCR reaction. Send purified product for sequencing using primers DR_215/DR_216.

[00951] Calculated OD600 readings were taken at T=0, 30, 60, 120, and 240 min after induction. All values after T0 are the average of 2 tubes. Results are shown in Table 34 and FIG. 16. The + indicates the cultures that received AtC, and the - indicates the cultures that did not receive any additional factors. FIG. 16 shows calculated OD600 values vs. time. The dashed lines represent the cultures that received 150ng/mL AtC at T=0. FIG. 16 shows the sprA gene that codes for the PepA1 toxin protein showed the largest reduction in viable 502a *Staphylococcus aureus* cells after 4 hours of growth post induction.

[00952] Specifically, FIG. 16 shows cell growth pre- and post-induction of four synthetic strains derived from *Staphylococcus aureus* 502a having a plasmid based inducible expression system comprising four different cell death gene candidates sprA1, 187-lysK, Holin, and sprG. The candidate cell death genes had been cloned behind an tetracycline inducible promoter on pRAB11 plasmids and transformed into *Staphylococcus aureus* 502a cells. Calculated OD600 readings were taken at T=0, 30, 60, 120, and 240 min after induction of AtC induced (+) strains illustrated by dashed lines (- - - -) and uninduced (-) strains indicated by solid lines (—) for BP_068 (502a pRAB11-Ptet-sprA1), BP_069 (502a pRAB11-Ptet-187lysK), BP_070 (502a pRAB11-Ptet-holin), and BP_071 (502a pRAB11-Ptet-sprG1) and compared to BP_001 (502a wt) in BHI media. Each of the induced (+) strains BP_068 (sprA1), BP_069

(187lysK) and BP_070 (holin) exhibited both (i) good cell growth pre-induction and (ii) significant inhibition of cell growth post-induction. BP_068 (+) exhibited the best inhibition of cell growth at each time point T=30, T=60, T=60, T=120 and T=240 min post-induction, so the sprA1 gene was selected for initial further development of a kill switch in *Staphylococcus aureus* 502a.

[00953] Table 34. Calculated OD600 at T=0, 30, 60, 120, and 240 min after induction as shown in FIG. 16

	Average OD600 Readings				
Strain +/- ind.	T0	T30 min	T60 min	T120 min	T240 min
68+	1.05	0.05	0	0	0
68-	1.05	1.45	2.05	3.4	5.7
69+	1	0.15	0	0.1	0
69-	0.95	1.25	1.75	2.8	5.5
70+	1	0.8	0.7	0.5	0.4
70-	0.9	1.3	1.8	2.9	5.6
71+	1	1.1	1.4	2.1	5.2
71-	1	1.5	2	3.1	5.8
502a+	1.1	1.15	1.45	2.1	3.7
502-	1.15	1.45	2.15	3.5	5.4

[00954] Table 35 below and FIG. 17 show colony forming units calculated from plate counts of diluted liquid culture samples. FIG 17 shows a bar graph showing difference in the colony forming units/mL between T=0 (gray) and 240 min (black).

[00955] Table 35. CFUs calculated from plate counts of diluted liquid culture samples.

	AtC	T0 (cfu/mL)	T240 (cfu/mL)
68	68+	2.85E+09	7.50E+01
	68-	3.12E+09	7.75E+09

69	69+	8.75E+09	6.30E+03
	69-	1.40E+09	1.10E+09
70	70+	5.25E+09	4.75E+04
	70-	6.05E+09	1.41E+11
71	71+	3.00E+09	1.04E+11
	71-	1.34E+09	2.69E+11
502	502a+	1.29E+09	2.07E+11
	502a-	1.45E+09	2.62E+11

[00956] This example investigated the effectiveness of multiple toxin genes when operably linked to an inducible promoter at disrupting cell viability when grown in complex rich media. Two native Staph toxins sprA and sprG, one chimeric phage toxin we have termed 187lysK, and one more phage holin toxin were tested using a plasmid based inducible expression system. The sprA1 gene that codes for the PepA1 toxin protein showed the largest reduction in viable 502a *Staphylococcus aureus* cells after 4 hours of growth post induction. The sprA1 gene was selected for initial further development of a kill switch in *Staphylococcus aureus* 502a.

[00957] Example 19. Induced Expression of GFP from the Genome in strain BP_076 (502a Δ sprA1::P_{tet}-gfp)

[00958] Overview. In this example the expression of green fluorescent protein (GFP) from the genome of a *Staphylococcus aureus* 502a variant strain (BP_076) was confirmed with quantitative polymerase chain reaction (qPCR). The *gfp* gene was integrated into the genome along with a tetracycline-inducible promoter (P_{tet}) and tetracycline repressor protein gene (*tetR*). The P_{tet}-*gfp* expression system was introduced into the genome *via* the suicide plasmid pIMAYz to allow for controllable expression of a recombinant gene. The wild-type strain (BP_001) served as the negative control and a strain carrying a high-copy plasmid with the same P_{tet}-*gfp* expression system served as the positive control. Due to its lower toxicity than tetracycline, anhydrotetracycline (aTc) was used to induce expression at 100 ng/mL.

[00959] Summarized Results. When comparing the t = 0 min samples of BP_055 and BP_076 to BP_001, the qPCR data shows minor GFP expression before induction

(indicating that P_{tet} is leaky); however, the expression fold change after induction is still clearly evident. Different expression patterns are seen between plasmid-based and integrated *gfp*. Integrated *gfp* shows a sustained increase in expression throughout the assay, whereas plasmid-based *gfp* shows a high upregulation at 30 minutes and nearly no expression at 90 minutes. The difference in expression between BP_076 and BP_055 is due to the copy number of *tetR* per cell in each strain. BP_076 has one copy per cell, whereas BP_055 has 300-500 copies depending on the number of plasmids in each cell. The high amount of total TetR protein present in the BP_055 culture clearly exceeded the amount of aTc used for induction by the end of the assay, which lead to repression of *gfp* expression.

[00960] Bacteria Strains and Materials.

[00961] Strains

BP_001 (*Staphylococcus aureus* 502a)

BP_055 (SA 502a, p229_pRAB11-Ptet-GFP)

BP_076 (SA 502a, Δ sprA1::Ptet-GFP)

[00962] Brain Heart Infusion (BHI) media, BHI + Chloramphenicol (10 μ g/mL) agar plates, Anhydrotetracycline (aTc) were employed.

[00963] Samples were RNA (1 mL culture): t = 0, 30 and 90 minutes.

[00964] Methods -Strain Construction

1. In order to make a modification in the genome of *Staph aureus Staphylococcus aureus*, we must first add the required genetic elements to a plasmid capable of making those modifications.
2. The plasmid backbone is an *E. coli* – *Staphylococcus aureus* shuttle vector called pIMAYz, and has chloramphenicol resistance, a low copy *E. coli* origin of replication, a low copy temperature sensitive *Staphylococcus aureus* origin of replication (permissible replication at 30°C, but not at 37°C), the secY toxin under the control of a Ptet promoter, and a lacZ gene for blue/white screening during integration into *Staphylococcus aureus*.
3. The plasmid was constructed using linear PCR products that were assembled into a circular construct using Gibson Assembly
 - a. Use primers DR_022/DR_023 to PCR amplify the backbone of the pIMAYz vector to linearize it for use in downstream assemblies. The background template DNA must be enzymatically digested with DpnI (NEB) per manufacturer's instructions prior to further use.

- b. Use primers DR_255/DR_241 to PCR amplify the tetR-Ptet-GFP region using the pRAB11 plasmid as the template.
 - c. Use primers DR_256/DR_257, and DR_240/DR_236 to PCR amplify 1kb regions from the *Staphylococcus aureus* 502a genome. These will be used as homology arms to target the region for integration into the *Staphylococcus aureus* genome.
 - d. These linear fragments are then assembled into a circular plasmid with the Gibson Assembly Master mix (NEB) per manufacturer's instructions and transformed into IM08B cells.
4. Once the sequence of the new plasmid DNA can be confirmed, 50 mL cultures are started to obtain a sufficient amount for transformation into *Staphylococcus aureus* 502a by electroporation.
5. Integration into *Staphylococcus aureus* by homologous recombination
 - a. Use between 1 and 5 micrograms of plasmid DNA to electroporate into *Staphylococcus aureus*. Recover at 37°C for 1 hour, and plate on BHI + 10 ug/mL chloramphenicol and 100 ug/mL x-Gal, and incubate overnight at 37°C.
 - b. The following day pick multiple blue colonies and start 5 mL BHI broth cultures at room temp, and allow them to grow in a rotary shaking unit for 12-20 hours.
 - c. Perform and plate serial dilutions (usually 10^{-4} - 10^{-6}) on BHI + 1ug/mL anhydrotetracycline (AtC) and 100 ug/mL X-gal. Incubate overnight at 37°C.
 - d. The following day, pick and screen white colonies by patching onto BHI, BHI + 1ug/mL anhydrotetracycline (AtC) and 100 ug/mL X-gal, and BHI + 10 ug/mL chloramphenicol and 100 ug/mL x-Gal agar plates to confirm chlor sensitivity and AtC resistance.
 - e. Colonies showing the desired phenotypes should be screened by PCR with primers DR_237/DR_238. Colonies that have taken the new genes should produce a 4.4 kb band, and colonies that have reverted back to wild type should have a 2.86 kb band. Several positive clones should be sequenced to verify the correct sequences, and one of the sequence verified clones to be picked for use in downstream experiments.

[00965] Cell Growth Procedure

1. Start overnight cultures of each strain in BHI broth media (5 mL) and incubate with agitation (37°C, 240 rpm). Add chloramphenicol (final concentration 10 µg/mL) to the media for BP_055.
2. Measure optical density (OD) of overnight culture and record.

The optical density (OD) of the cultures was measured at 630 nm, fresh media served as the blank. The OD of the overnight cultures is denoted as the initial OD. The inoculum transferred to 5 mL of fresh media reduced the OD to 0.05 so that the new cultures would be in the exponential growth phase two hours after inoculation, as shown in Table 36.

[00966] Table 36 . OD of cultures for P_001, BP_055 and BP_076

Strain	Initial OD	Inoculum for 5 mL [µL]	OD at 2hr
BP_001	8.2	30.5	1.01
BP_055	9.1	27.5	0.88
BP_076	8.7	28.7	1.01

3. Dilute overnight cultures to 0.05 OD in fresh BHI (5 mL) in 2× 14 mL culture tubes per culture; again add chloramphenicol to the BP_055 cultures.
4. Incubate with agitation (37°C, 240 rpm) until OD reaches 0.5 – 1 (“2hr culture”).
5. Remove 1 mL of culture for t = 0 min RNA samples and transfer them to 1.5 mL microtubes. Spin down the samples (16,000× g, 1 min, RT), aspirate off supernatant and resuspend the pellet in 200 µL RNALater. Allow them to incubate for a few minutes at room temperature (RT) and then store at -20°C.
6. Add aTc (4 µL, 100 µg/mL) to first 14 mL culture tube for each strain. Add 4 µL 100% ethanol to second tube for each strain as induction controls (the aTc was solvated in 100% ethanol).
7. Incubate the cultures with agitation (37°C, 240 rpm) until other sampling timepoints.
8. Repeat RNA sampling at t = 30 and 90 mins, measure OD at t = 90 mins.

qPCR Sample Processing and Data Analysis

RNA was extracted from frozen cell pellets stored in RNALater using Ambion RiboPure Bacteria Kit per protocols in example above. The *gfp* expression level was normalized to

the housekeeping gene *gyrB* and quantitated using the $\Delta\Delta C_t$ method, see the primer sequences in Table 37.

[00967] Table 37 : Sequences of qPCR primers.

Target	Database Number	Sequence
<i>gyrB</i>	BP 802	5'-TTGGTACAGGAATCGGTGGC (SEQ ID NO: 212)
<i>gyrB</i>	BP 803	5'-TCCATCCACATCGGCATCAG (SEQ ID NO: 213)
<i>gfp</i>	BP 195	5'-CTGTCCACACAATCTGCCCT (SEQ ID NO: 292)
<i>gfp</i>	BP 196	5'-TGCCATGTGTAATCCCAGCA (SEQ ID NO: 293)

[00968] Primer sequences used for plasmid and strain construction are shown in Table 38.

[00969] Table 38. Primers used for plasmid and strain construction

Primer Name	ssDNA sequence (5'-3')
DR_022	Caagcttatcgataccgtcgacctc (SEQ ID NO: 294)
DR_023	Gggatccactagttagagcgg (SEQ ID NO: 295)
DR_237	GCAACTGGTACATCACAATTGGTACTCTCAC (SEQ ID NO: 296)
DR_238	GACCACGCATACCTATCTATAAACGGACAATG (SEQ ID NO: 297)
DR_255	GTCCAATTAGATGGCATGTAAGTGGGCAGTGTCTTAAAAAATCG (SEQ ID NO: 298)
DR_241	CAGGCCAATTGTCATAGAGCCGGATGTGCTGCAAGGCGATTAAGTT GGGTAACG (SEQ ID NO: 299)
DR_256	GTTACATGCCATCTAATTGGACAAATTCTATGAGAGTAGATTTTG (SEQ ID NO: 300)
DR_257	GCCAAATCGCTTTCGTGTATACGATTCCCAGTC (SEQ ID NO: 301)

DR_240	GGCTCTATGCCAAATTGGCCTGATGAGTTC (SEQ ID NO: 302)
DR_236	gctctagaactagtgatcccGGCGATTTTATTGTGACAAGAGACTGAAGAGC (SEQ ID NO: 303)

FIG. 19 shows a map of the genome for Strain BP_076 (SA 502a, Δ sprA1::Ptet-GFP).

FIG. 20 shows a map of plasmid constructed for making genomic integration in *Staphylococcus aureus*.

[00970] Results. The $t = 0$ samples of both strains carrying the P_{tet} -*gfp* system showed some GFP expression before induction, Table 2 shows the Ct values of the three investigated strains at $t = 0$. The wild-type strain BP_001 amplification curve crossed the threshold (0.4) after 30 cycles, which may be attributed to some form of unspecific amplification or primer dimer formation.

Table 39 shows the Cycles to Threshold (Ct) values prior to expression induction for the wild-type strain BP_001, plasmid based P_{tet} -*gfp* BP_055 carry strain and P_{tet} -*gfp* genetically modified strain BP_076 are shown. The threshold was set to 0.4.

[00971] Table 39. Cycles to Threshold (Ct) values prior to induction for BP_001, BP_055 and BP_076

Strain	BP_001	BP_055	BP_076
Ct Value	33.65 $\pm$ 0.61	22.99 $\pm$ 0.06	23.09 $\pm$ 0.10

[00972] The basal expression level of GFP was accounted for in the $\Delta\Delta$ Ct calculations by normalizing the experimental timepoints ($t = 30$ min, 90 min) to the control timepoint ($t = 0$) for each strain individually. The expression levels of GFP determined by qPCR are displayed below in FIG.18. FIG. 18 shows GFP expression fold change of induced (+) and uninduced (-) subcultures of *Staphylococcus aureus* strains BP_001, BP_055 and BP_076. Different expression patterns are seen between plasmid-based and integrated *gfp*. Integrated *gfp* shows a sustained increase in expression throughout the assay, whereas plasmid-based *gfp* shows a high upregulation at 30 minutes and nearly no expression at 90 minutes. The induced subculture (+) and uninduced subculture (-) for all three strains show expression induction dependency on the presence of aTc and the P_{tet} -*gfp* expression system. As expected, BP_001 showed

no expression throughout the experiment. The expression of GFP in BP_076 increased throughout the experiment, demonstrating expression from the genome of *Staphylococcus aureus* 502a. The expression pattern determined for BP_055 can be attributed to less than ideal experimental design; however, it did fulfill its purpose as a positive control for induction. BP_055 carries the P_{tet} -gfp expression system on the plasmid pRAB11, a high-copy plasmid. Each plasmid has two TetR protein binding sites, which repress expression of GFP in the absence of aTc. Within 30 minutes of induction the high number of plasmids multiplied by cell count resulted in a ca. 1300 fold upregulation in GFP expression, confirming aTc was in an active form during the assay. One might expect that the expression level of GFP would be even higher at 90 minutes, but the data shows nearly no expression (ca. 7 fold upregulation compared to $t = 0$). This is not surprising given the total number of TetR proteins present in the culture at $t = 90$ minutes. The amount of aTc was not enough to inhibit repression by TetR at the 90-minute timepoint, resulting in nearly no expression. Gene expression from a molecularly modified strain of *Staphylococcus aureus* 502a was confirmed by qPCR analysis of tetracycline induced GFP expression.

[00973] Example 20. Candidate Serum Responsive Promoters screened by RNA seq to detect up-regulation

[00974] In this experiment, RNA sequencing of 502a *Staphylococcus aureus* variant strain BP_001 WT when grown in human serum compared to TSB was performed in order to gain a holistic understanding of the transcriptional changes that occur within the microorganism upon entry into the circulatory system. RNA sequencing was performed on samples collected from laboratory growth medium and human serum.

[00975] A culturing (growth assay) in TSB with or without human serum was performed as follows. *S. aureus* 502a cells were struck out from a cryo stock on a tryptic soy broth (TSB) agar plate with 5% sheep's blood and grown overnight (37°C). The following day five single colonies were used to inoculate 5 mL of TSB in a 14 mL culture tube and grown overnight with agitation (37°C, 240 rpm). The next morning 50 mL of TSB were transferred to a 250 mL flask and warmed to 37°C. The OD₆₀₀ of the overnight culture was measured (OD₆₀₀ = 6.0) and used to inoculate (416 µL) the warmed TSB to an OD₆₀₀ of 0.05. This culture grew for ca. two hours (37°C, 100 rpm) and reached an OD₆₀₀ of 1.24. During this time a 50 mL aliquot of human serum was placed in the 37°C

incubator to thaw and warm, fresh TSB was also warmed. Using a serological pipette, 15 mL of culture were transferred to a 15 mL Falcon tube and centrifuged (RT, 2000× g, 10 min). The supernatant was decanted, the pellet was resuspended in sterile PBS (15 mL) and centrifuged (RT, 2000× g, 10 min). The supernatant from the wash step was decanted and the pellet was resuspended in sterile PBS (7.5 mL), doubling the OD₆₀₀ of the inoculum to 2.48. The PBS suspension was used to inoculate the TSB and serum culture samples at an OD₆₀₀ of 0.05 (202 µL per 10 mL medium).

[00976] RNA sequencing sample preparation was performed as follows.

[00977] The $t = 0$ min samples (3×) were each 1 mL of the original 50 mL starter culture prior to washing. At the allotted timepoint, the culture tubes were removed from the incubator and placed in an ice water bath for 5 minutes and then centrifuged (4°C, 2000× g, 10 min). The supernatant was decanted, the pellet was resuspended in 1 mL ice-cold sterile PBS and transferred to microtubes. The suspensions were centrifuged (4°C, 6000× g, 3 min), the supernatant was aspirated off and the pellets were resuspended in RNAlater. The RNAlater suspensions were stored at −20°C.

[00978] The samples were removed from the −20°C freezer for RNA extraction and allowed to thaw at RT. The cells were pelleted (RT, 16000× g, 1 min), the supernatant was aspirated off and the cells were then washed with PBS – washing helped remove carryover from the serum. To wash the cells, the pellets were resuspended in PBS and centrifuged (RT, 16000× g, 1 min), the supernatant was discarded. The RNA was extracted using Invitrogen's RiboPure Bacteria Kit following the manufacturer's instructions. The extracted RNA was then DNase I treated and ethanol precipitated. Per the sequencing firm's request the samples were sent as pellets in ethanol on dry ice.

[00979] From the total RNA samples, the ribosomal RNA molecules were depleted using the Ribo-Zero rRNA Removal Kit for Bacteria (Illumina). The quality of the RNA samples was analyzed on a Shimadzu MultiNA microchip electrophoresis system and then fragmented using ultrasound (4 pulses, 30 s, 4°C). An adapter was ligated to the 3' end of the molecules to enable first strand cDNA synthesis with M-MLV reverse transcriptase. The cDNA was purified and a 5' Illumina TruSeq adapter ligated to the 3' end of the antisense cDNA. The cDNA was then amplified by PCR using a high fidelity polymerase, the concentration after amplification was 10-20 ng/µL. The cDNA samples were then barcoded according to the growth condition they represented, purified using a Agencourt AMPure XP kit (Beckman Coulter Genomics) and analyzed by capillary electrophoresis. The cDNA was then pooled, the pool

covered 200 to 500 bp molecules.

[00980] For Illumina NextSeq the primers used for PCR amplification were designed for TruSeq sequencing following Illumina's instructions. The cDNA was sequenced on an Illumina NextSeq 500 system using 75 bp read length. The differential expression of genes was analyzed *via* DESeq2 using SARTools.

[00981] Results for upregulated genes by RNA sequencing are shown in the Table 40; t= time in minutes after exposure to human serum.

[00982] Table 40. Genes in *Staphylococcus aureus* 502a WT upregulated upon exposure to human serum by RNAseq

Gene		t = 30 Serum vs t = 0	t = 30 Serum vs t = 30 TSB	t = 90 Serum vs t = 0	t = 90 Serum vs t = 90 TSB
gene name	gene number	fold change	fold change	fold change	fold change
isdB	CH52_00245	479.653	471.648	2052.474	1240.112
sbnB	CH52_05135	158.756	44.41	310.08	130.622
isdC	CH52_00235	93.006	56.211	173.376	149.117
sbnA	CH52_05140	88.832	37.808	143.558	93.474
srtB	CH52_00215	73.135	47.421	143.059	170.578
sbnE	CH52_05120	70.475	50.083	190.255	171.279
sbnD	CH52_05125	66.84	52.434	187.025	224.017
isdI	CH52_00210	65.951	53.426	115.302	118.724
heme ABC transporter 2	CH52_00225	65.024	43.415	117.603	135.956
sbnC	CH52_05130	63.092	51.306	162.927	147.385
heme ABC transporter	CH52_00230	60.967	40.137	125.227	196.142
isd ORF3	CH52_00220	51.262	35.978	97.439	119.584
sbnF	CH52_05115	43.997	44.31	129.516	127.889
alanine dehydrogenase	CH52_11875	43.589	20.237	304.444	NA
HarA	CH52_10455	43.215	28.041	114.425	117.787
sbnG	CH52_05110	42.446	34.095	133.373	120.433
diaminopimelate decarboxylase	CH52_05105	32.541	25.864	102.838	141.629
iron ABC transporter	CH52_05145	31.417	19.576	44.885	47.226
threonine dehydratase	CH52_11880	24.559	20.237	NA	NA

isdA	CH52_00240	21.471	40.712	44.477	115.432
siderophore ABC transporter	CH52_05150	NA	NA	33.201	37.267
sbnI	CH52_05100	NA	22.602	101.548	89.778
SAM dep Mettrans	CH52_04385	NA	NA	75.292	25.847

[00983] Several genes were found to be upregulated greater than 20 -fold after exposure to human serum at t=30 min compared to t=0, or compared to t=30 in TSB, by RNA sequencing including isdB, sbnB, isdC, sbnA, srtB, sbnE, sbnD, isdI, heme ABC transporter 2, heme ABC transporter 2, heme ABC transporter, isd ORF3, sbnF, alanine dehydrogenase, HarA, sbnG, diaminopimelate decarboxylase, iron ABC transporter, threonine dehydratase, isdA, and sbnI.

[00984] Several genes were upregulated greater than 50 -fold after exposure to human serum at t=30 min compared to t=0, or compared to t=30 in TSB, by RNA sequencing including isdB, sbnB, isdC, sbnA, srtB, sbnE, sbnD, isdI, heme ABC transporter 2, heme ABC transporter 2, heme ABC transporter, isd ORF3. Genes upregulated greater than 100 -fold after exposure to human serum at t=30 min compared to t=0, or compared to t=30 in TSB, by RNA sequencing include isdB, and sbnB,

[00985] Several genes were upregulated greater than 100-fold after exposure to human serum at t=90 min compared to t=0, or compared to t=90 in TSB, by RNA sequencing including isdB, sbnB, isdC, sbnA, srtB, sbnE, sbnD, isdI, heme ABC transporter 2, heme ABC transporter 2, heme ABC transporter, isd ORF3, sbnF, alanine dehydrogenase, HarA, sbnG, diaminopimelate decarboxylase, isdA.

[00986] Preferred upregulated genes in *Staphylococcus aureus* 502a when exposed to serum include isdB gene CH52_00245, srtB gene CH52_00215, heme ABC transporter2 gene CH52_00215, and HarA gene CH52_00215.

[00987] Several *Staphylococcus aureus* 502aWT genes were found to be downregulated when exposed to human serum by RNA sequencing as shown in Table 41 and Table 42.

[00988] Table 41. Genes in *Staphylococcus aureus* 502a WT downregulated upon exposure to human serum at 30 min by RNAseq

gene name	gene number	t = 30 Serum vs t = 0	t = 30 Serum vs t = 30 TSB
		fold change	fold change
phosphoribosylglycinamide formyltransferase	CH52_00525	-4.307	-2.001
phosphoribosylaminoimidazole synthetase	CH52_00530	-4.271	-2.063
amidophosphoribosyltransferase	CH52_00535	-4.131	-2.117
phosphoribosylformylglycinamidine synthase	CH52_00540	-4.046	-2.244
phosphoribosylformylglycinamidine synthase	CH52_00545	-3.498	-2.215
phosphoribosylaminoimidazole-succinocarboxamide	CH52_00555	-3.345	-2.134
trehalose permease IIC	CH52_03480	-3.338	-2.401
DeoR family transcriptional regulator	CH52_02275	-2.55	-2.171
phosphofructokinase	CH52_02270	-2.464	-1.984
PTS fructose transporter subunit IIC	CH52_02265	-2.042	-1.806
galactose-6-phosphate isomerase	CH52_07975	NA	-2.137

[00989] Table 42. Genes in *Staphylococcus aureus* 502a WT downregulated upon exposure to human serum at 90 min by RNAseq

gene name	gene number	t = 90 Serum vs t = 0	t = 90 Serum vs t = 90 TSB
		fold change	fold change
NarZ	CH52_07000	-5.012	-3.989

phosphoribosylglycinamide formyltransferase	CH52_00525	-3.737	-1.680
trehalose permease IIC	CH52_03480	-3.279	-4.381
NarH	CH52_07005	-3.265	NA
alkylhydroperoxidase	CH52_06615	-3.211	-3.573
NarT	CH52_07045	-3.108	-3.680
hypothetical protein	CH52_04875	-2.911	-3.396
DeoR trans factor	CH52_02275	-2.245	-3.322
PTS fructose transporter subunit IIC	CH52_02265	-2.211	-4.474
lysophospholipase	CH52_02680	-1.837	-3.000
protein disaggregation chaperon	CH52_01005	-0.009	-2.989
alkylhydroperoxidase	CH52_06615	NA	-3.573
phosphofructokinase	CH52_02270	NA	-3.878

[00990] Several genes in *Staphylococcus aureus* 502a were downregulated at least 2 fold after t=30 or t=90 minutes in serum compared to t=0 or in TSB including phosphoribosylglycinamide formyltransferase gene CH52_00525, trehalose permease IIC gene CH52_03480, DeoR family transcriptional regulator gene CH52_02275, phosphofructokinase gene CH52_02270, and PTS fructose transporter subunit IIC gene CH52_02265.

[00991] **Example 21. Kill Switch construction**

[00992] For this experiment, a serum responsive kill switch cassette was designed and constructed for the purpose of making a strain of *Staphylococcus aureus*

(SA) 502a that is unable to grow in serum or blood. We based this cassette around the endogenous sprA1 toxin antitoxin system in SA. This is a type I T/AT system where the toxin is a small membrane porin peptide (PepA1) that is translationally repressed by an antisense RNA. The antisense RNA binds to the 5' UTR of sprA1 covering the RBS and blocking its ability to bind to the single stranded mRNA and synthesize the protein.

[00993] The design of this kill switch changes the promoter region that drives the expression of the PepA1 toxin from its endogenous system to one that is highly upregulated when the organism is cultured in human serum. This construct was made with the sbnA promoter from SA 502a. For this kill switch, the promoter region was not changed for the antisense RNA, but additional versions of kill switches are in progress that will have this region changed as well to promoters that have been identified to be highly upregulated during growth in normal complex media, but highly repressed or down regulated when the organism is grown in blood or serum. This should make it even easier to overcome the antitoxin suppression of sprA1 in blood or serum conditions.

[00994] To test the functionality of the kill switch, the expression of the PepA1 toxin was induced by taking a culture that was growing at early exponential phase in complex media, tryptic soy broth (TSB), and changing the growth media to human serum. The OD was monitored and serial dilutions to plate were performed and CFUs were counted to monitor the number of viable cells in the culture and compare it to wild type SA 502a grown under the same conditions. FIG. 21 shows a map of PsbnA-sprA1 Kill Switch in *Staphylococcus aureus* 502a genome.

[00995] The methods used for plasmid construction, oligos, protocol for making changes in *Staphylococcus aureus* 502a genome using homologous recombination, and Kill Assay are shown below.

[00996] Strains

502a – *Staphylococcus aureus* wild type

BP_011 – 502a Δ sprA1-sprA1(AS)

BP_084 – 502a Δ PsprA::PsbnA

[00997] In this experiment BP_011 has both the sprA1 toxin gene and sprA1 antitoxin region knocked out, because it was considered to be easier to "cure" the KO by integrating the kill switch into that site than to do the integration directly into the wild type 502a. This is because the system used for integrations, i.e. homologous recombination, relies on segments of homology between the inserted gene and the

chromosomal target to dictate the location of the integration, and it was felt the endogenous sprA1 toxin/antitoxin might interfere with the integration if present in the genome. The BP_011 strain is the parent of the kill switch strain BP_084. The BP-011 strain was included in this experiment as a control.

[00998] Plasmid Construction

- 1) PCR amplify homology regions from SA 502a genome
 - a. Upstream Homology Arm – DR_233/DR_296
 - b. Downstream Homology Arm – DR_280/DR_236
- 2) PCR amplify PsbnA-sprA1 from synthesized linear DNA fragment from IDT
 - a. PsbnA-sprA1 – DR_297/DR_228
- 3) PCR amplify pIMAYz backbone vector
 - a. DR_022/DR_023
- 4) Gel purify all fragments with Qiagen kit per manufactures instructions
- 5) Assemble linear DNA fragments into circular plasmid and transform into electrocompetent IM08B *E. coli* cells per the manufacturer's instructions
- 6) Perform colony PCR to screen colonies for fully assembled plasmid
 - a. DR_117/DR_228 (1571bp fragment)
- 7) Pick multiple positive colonies, grow culture overnight and sequence the plasmid to confirm there are no mutations in the newly assembled plasmid
- 8) Transform sequence confirmed plasmid into electrocompetent SA 502a and follow protocol for making edits in SA genome using homologous recombination
- 9) Screen final colonies by PCR for integrant with the primer pair DR_303/DR_304
 - a. Send PCR product for sequence confirmation if correct band size is observed.

[00999] Table 43. Oligo Sequences used in plasmid construction

Primer Name	5'-3' DNA sequence
DR_233	cgacgggtatcgataagcttgGCCACTGGCGTCAAATACTGTAATGAAGAATG
3	(SEQ ID NO: 330)

DR_29 6	CATCTAATTGGACAAATTCTATGAGAGTAGATTTTGTTAATTAA G (SEQ ID NO: 331)
DR_28 0	GTAGACGCAATACAAAATAGGTGACATATAGCCGCACC (SEQ ID NO: 332)
DR_23 6	gctctagaactagtggatcccGGCGATTTTATTGTGACAAGAGACTGAAGAG C (SEQ ID NO: 333)
DR_29 7	CATAGAATTTGTCCAATTAGATGTCCCACTACATCCTGCTAAAAC AAGTAGGAAAGC (SEQ ID NO: 334)
DR_22 8	CTATTTTGTATTGCGTCTACTTAGCCAATAAG (SEQ ID NO: 335)
DR_02 2	Caagcttatcgataccgctcgacctc (SEQ ID NO: 336)
DR_02 3	Gggatccactagttctagagcgg (SEQ ID NO: 337)
DR_30 3	CAAGCCACCAAAGCACGTGCCTATTTGCC (SEQ ID NO: 338)
DR_30 4	CAGTGAAATAGATAGATTGGTTGAAAAACAATCTTCAAAAGTCG GACG (SEQ ID NO: 339)

[001000] The protocol used for making changes in *Staphylococcus aureus* 502a genome using homologous recombination is shown below.

Materials

- BHI agar (Chloramphenicol 10ug/mL) (X-Gal 100ug/mL)
- BHI agar (AnhydroTet 1ug/mL) (X-Gal 100ug/mL)

- BHI agar
- BHI broth
- Primers to screen colonies after primary and secondary recombination events

Protocol

1. Prepare a highly concentrated pIMAYz integration plasmid. ~25mL overnight culture spun down into (4) 2x volumes of the miniprep protocol. This can be purified through 2 columns if desired, and performed to maximize yield of DNA. Elutions should be pooled and concentrated using the Zymo concentrator kit performed to maximize concentration.
2. Use up to 5uL of concentrated plasmid from above to transform 502a using the labs optimized electroporation protocol.
3. Recover cells for 1 hr at 30°C in shaker
4. Plate entire recovery mixture between 3-4 BHI (Chlor 10, X-Gal 100) agar plates. Incubate 1 plate at 30°C and the rest at 37°C overnight (make sure incubator is at 37C or above)
5. Screen blue colonies on the plates for the presence of circular plasmid using primers DR_116/DR_117. The primers are flanking the multiple cloning site in pIMAYz, and for the 30C plates will produce a band the same size as the homology arms plus any region being integrated. The 37°C plates should not produce any band.
6. The blue colonies on the 37°C plates should be screened for the integrated plasmid into the genome using primers that bind outside the homology arms. Each primer should be paired with either DR_116 or DR_117. This will confirm that the plasmid is integrated into the proper location in the genome.
7. If no colonies on the 37°C plates produce bands indicating the plasmid has been integrated, colonies showing a plasmid band on the 30°C plates can be diluted and plated on BHI agar (Chlor 10, X-Gal 100) and incubated at 37°C. Repeat steps 5-6 to rescreen the new colonies for integration.
8. If PCR shows integrated plasmid, pick a couple colonies, if possible pick clones that have integrated each way. Grow overnight (~16hr) in 5mL BHI broth in room temp shaker.
9. Dilute to 10^{-5} and 10^{-6} and plate 50uL on BHI agar (AnhydroTet 1ug/mL, X-Gal 100ug/mL). Incubate plates overnight at 37°C.

10. Patch white colonies to BHI agar (Chlor 10uG/mL, X-Gal 100), BHI agar (AnhydroTet 1ug/mL, X-Gal 100ug/mL), BHI agar to screen for resistance to anhydrotet and sensitivity to chloramphenicol. Colonies with both phenotypes should be picked from the BHI agar plate and screened for the knock out or knock in. At least one of the primers used to screen the final genotype should bind outside the regions used as homology arms.
11. Streak plate from patch plate of several positive clones, perform HF PCR using primers that bind outside the homology arms, and send for sequencing. Incubate plates overnight at 37°C.
12. Pick at least 3 colonies from struck out plates and perform colony PCR to confirm genotype. If PCR's are all positive, the plate is used to create strain stocks and a new strain number is assigned.

[001001] The kill assay used for preliminary evaluation of the synthetic PsbnA-sprA1 Kill Switch in *Staphylococcus aureus* 502a genome is shown below.

[001002] Kill Assay

- 1) Start 5mL TSB cultures of strains to be tested and wild type control strain and grow overnight at 37°C in an incubator with orbital shaking at 250 RPM
- 2) The following day perform 1:100 dilutions into fresh TSB media and allow the cultures to grow for 2 hours.
- 3) Take an OD600 reading and record the values. Calculate the volume of cell culture required to inoculate 5mL cultures to an OD of 0.05. Inoculate new cultures with calculated volume into prewarmed media (TSB/serum)
- 4) Continue to grow cultures at 37°C. Perform serial dilutions and plate several cell dilutions on BHI or TSB agar plates. Incubate the plates overnight at 37°C and count the colonies on each plate after they appear (>16hr).

[001003] Preliminary results using PsbnA-sprA1 Kill Switch in *Staphylococcus aureus* 502a genome showed there was no difference in growth curves between KS and wild-type under normal growth conditions in TSB, as desired. Recorded colony counts are shown in Table 44 and FIG. 23.

[001004] Table 44. Recorded colony counts after 180 min when exposed to human serum

Strain		Recorded Colony Count			
		t = min	t = 0 min	t = 45min	t = 90 min t = 180 min
BP_011	TSB		188×10^4	409×10^4	30×10^6 68×10^7
	Serum		560×10^3	76×10^4	63×10^5 5×10^7
502a	TSB			305×10^4	199×10^5 89×10^6
	Serum			305×10^4	35×10^5 6×10^7
BP_084	TSB		220×10^4	75×10^5	77×10^5 135×10^6
	Serum		62×10^4	180×10^4	34×10^5 157×10^4

[001005] As shown in Table 44 and FIG. 23, after three hours of exposure to human serum, the *Staphylococcus aureus* KS strain BP_084 having the kill switch incorporated to the genome had fewer colonies than the wild-type strain by a factor of about 1000.

[001006] The calculated cfu/ml was found by taking the number of colonies counted*dilution factor*20 (to account for 50uL being plated from each dilution) as shown in Table 45.

[001007] Table 45. Calculated cfu/mL in Human Serum and TSB

Strain		Calculated CFU/mL			
	t = min	0	45	90	180
BP_011	TSB	37600000	81800000	600000000	13600000000
	Serum	11200000	15200000	126000000	1000000000
502a	TSB		61000000	398000000	1780000000
	Serum		61000000	70000000	1200000000
BP_084	TSB	44000000	150000000	154000000	2700000000
	Serum	12400000	36000000	68000000	31400000

[001008] Using the data in Table 45, the cfu/mL of the kill switch strain was compared to wild type 502a. After 3 hours post serum induction, the strain harboring the integrated kill switch *Staphylococcus aureus* KS strain BP_084 (502a Δ PsprA::PsbA) showed a survival rate of 2.61%, which corresponds to a 97.39% reduction in viable cells compared to the wild type in serum.

[001009] Also as shown in Table 45 after three hours of exposure to human serum, the *Staphylococcus aureus* KS strain BP_084 having the kill switch

incorporated to the genome exhibited the survival percentage of $BP_084(\text{serum})/BP_084(\text{TSB}) \times 100 = 1.16\%$ survival percentage. Therefore, when exposed to human serum the *Staphylococcus aureus* KS strain BP_084 (502a $\Delta P_{sprA}::P_{sbnA}$) cells at 3 hours post-induction exhibited $100\% - 1.16\% = 98.84\%$ measurable average cell death compared to the same BP_084 cells in TSB.

[001010] The synthetic microorganism BP_084 comprising the kill switch molecular modification incorporated to the genome exhibited desired growth properties under normal conditions, but significantly reduced cell growth when exposed to human serum.

[001011] Example 22. Kill Switch construction with Expression Clamp

[001012] Kill switch construction with expression clamp will be performed as follows. In prior examples, certain genes were identified that are up or down regulated in *Staphylococcus aureus* when exposed to human serum and blood. For example, *isdB* is selected as a promoter that is significantly upregulated a blood and serum responsive promoter. Also, *clfB* is selected as a second promoter for use in an expression clamp that is active in the absence of serum or blood, but is downregulated in the presence of serum or blood.

[001013] In prior examples, an endogenous toxin in *Staph aureus* was identified that when significantly upregulated, kill the cell. For example, *sprA1* toxin is selected as a cell death gene.

[001014] By using stitch PCR and Gibson assembly, operons are constructed that use the promoter region responsible for upregulating serum/blood genes in *Staphylococcus aureus* to drive the expression of the *sprA1* toxin, and using the promoter regions responsible for downregulating serum/blood genes in *Staphylococcus aureus* to drive the expression of the *sprA1_{AS}*. FIG. 22 shows a cartoon of kill switch construction using serum and blood responsive promoter *isdB* operably linked to *sprA1* cell death gene and a second promoter *clfB* operably linked to *sprA_{AS}* to prevent leaky expression of the toxin in the absence of blood or serum. This cassette will be integrated into the native *sprA1* location in the genome of *Staphylococcus aureus* 502a by homologous recombination, using the same technique described in previous examples.

[001015] To confirm utility, kill assay experiments will be performed using synthetic *Staphylococcus aureus* 502a to determine functionality of Kill Switch under various culture conditions and dermal assays in the absence and presence of blood or

serum. The synthetic *Staphylococcus aureus* 502a will exhibit good growth under dermal or mucosal conditions, but will exhibit significantly reduced growth or cell death when exposed to blood or serum. It is contemplated that the colonized synthetic *Staphylococcus aureus* 502a will thus be safe to administer to a subject because it will be unable to survive or reproduce under systemic conditions. It is also contemplated that the synthetic *Staphylococcus aureus* 502a will be able to durably occupy a vacated niche in a host microbiome created by decolonization of a *Staphylococcus aureus* strain such as MRSA.

WHAT IS CLAIMED IS:

1. A synthetic microorganism, comprising a recombinant nucleotide comprising at least one kill switch molecular modification comprising
 - a first cell death gene operatively associated with
 - a first regulatory region comprising an inducible first promoter, wherein the first inducible promoter exhibits conditionally high level gene expression of the recombinant nucleotide in response to exposure to blood, serum, or plasma of at least three fold increase of basal productivity.
2. The synthetic microorganism of claim 1, wherein the synthetic microorganism further comprises
 - at least a second molecular modification (expression clamp) comprising
 - an antitoxin gene specific for the first cell death gene, wherein the antitoxin gene is operably associated with
 - a second regulatory region comprising a second promoter which is active (constitutive) upon dermal or mucosal colonization or in a complete media, but is not induced, induced less than 1.5-fold, or is repressed after exposure to blood, serum or plasma for at least 30 minutes.
3. The method of claim 1 or 2, wherein the synthetic microorganism is derived from a target microorganism having the same genus and species as an undesirable microorganism.
4. The synthetic microorganism of claim 1 or 2, wherein the first promoter is upregulated by at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold within at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, or at least 360 min following exposure to blood, serum, or plasma.
5. The synthetic microorganism of claim 1 or 2, wherein the first promoter is not induced, induced less than 1.5 fold, or is repressed in the absence of blood, serum, or plasma.

6. The synthetic microorganism of claim 2, wherein the second regulatory region comprising a second promoter is active upon dermal or mucosal colonization or in TSB media, but is repressed at least 2 fold upon exposure to blood, serum or plasma after a period of time selected from the group consisting of the group consisting of at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, and at least 360 min.
7. The synthetic microorganism of any one of claims 1 to 6, wherein measurable average cell death of the synthetic microorganism occurs within at least a preset period of time following induction of the first promoter.
8. The synthetic microorganism of claim 7, wherein the measurable average cell death occurs within at least a preset period of time selected from the group consisting of within at least 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, or 360 min minutes following exposure to blood, serum, or plasma.
9. The synthetic microorganism of claim 8, wherein the measurable average cell death is at least a 50% cfu, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% cfu count reduction following the preset period of time.
10. The synthetic microorganism of any one of claims 1 to 9, wherein the kill switch molecular modification reduces or prevents infectious growth of the synthetic microorganism under systemic conditions in the subject.
11. The synthetic microorganism of claim 1 or 2, wherein the at least one molecular modification is integrated to a chromosome of the synthetic microorganism.
12. The synthetic microorganism of claim 3, wherein the target microorganism is susceptible to at least one antimicrobial agent.
13. The synthetic microorganism of claim 12, wherein the target microorganism is selected from a bacterial, fungal, or protozoal target microorganism.

14. The method of claim 13, wherein the target microorganism is a bacterial species capable of colonizing a dermal and/or mucosal niche and is a member of a genus selected from the group consisting of *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Pseudomonas*.

15. The synthetic microorganism of claim 14, wherein synthetic microorganism is derived from a *Staphylococcus aureus* strain.

16. The synthetic microorganism of claim 15, wherein the cell death gene is selected from the group consisting of *sprA1*, *sprA2*, *kpn1*, *sma1*, *sprG*, *relF*, *rsaE*, *yoeB*, *mazF*, *yefM*, or lysostaphin toxin gene.

17. The synthetic microorganism of claim 16, wherein the cell death gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 122, 124, 125, 126, 127, 128, 274, 275, 284, 286, 288, 290, 315, and 317, or a substantially identical nucleotide sequence.

18. The synthetic microorganism of claim 16 or 17, wherein the inducible first promoter comprises or is derived from a gene selected from the group consisting of *isdA* (iron-regulated surface determinant protein A), *isdB* (iron-regulated surface determinant protein B), *isdG* (heme-degrading monooxygenase), *hlgA* (gamma-hemolysin component A), *hlgA1* (gamma-hemolysin), *hlgA2* (gamma-hemolysin), *hlgB* (gamma-hemolysin component B), *hrtAB* (heme-regulated transporter), *sbnC* (luc C family siderophore biosynthesis protein), *sbnD*, *sbnI*, *sbnE* (lucA/lucC family siderophore biosynthesis protein), *isdI*, *lrgA* (murein hydrolase regulator A), *lrgB* (murein hydrolase regulator B), *ear* (Ear protein), *fhuA* (ferrichrome transport ATP-binding protein fhuA), *fhuB* (ferrichrome transport permease), *hly* (phospholipase C), heme ABC transporter 2 gene, heme ABC transporter gene, *isd* ORF3, *sbnF*, alanine dehydrogenase gene, diaminopimelate decarboxylase gene, iron ABC transporter gene, threonine dehydratase gene, siderophore ABC transporter gene, SAM dep Metrans gene, *HarA*, *spIF* (serine protease SplF), *spID* (serine protease SplD), *dps* (general stress protein 20U), SAUSA300_2617 (putative cobalt ABC transporter, ATP-binding protein), SAUSA300_2268 (sodium/bile acid symporter family protein), SAUSA300_2616

(cobalt family transport protein), *srtB* (Sortase B), *sbnA* (probable siderophore biosynthesis protein sbnA), *sbnB*, *sbnG*, *leuA* (2-isopropylmalate synthase amino acid biosynthetic enzyme), *sstA* (iron transport membrane protein), *sirA* (iron ABC transporter substrate-binding protein), *isdA* (heme transporter), and *spa* (Staphylococcal protein A).

19. The synthetic microorganism of claim 18, wherein the first promoter comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, and 163, or a substantially identical nucleotide sequence thereof.

20. The synthetic microorganism of any one of claims 15 to 19, wherein the antitoxin gene encodes an antisense RNA sequence capable of hybridizing with at least a portion of the first cell death gene.

21. The synthetic microorganism of claim 20, wherein the antitoxin gene is selected from the group consisting of a *sprA1* antitoxin gene, *sprA2* antitoxin gene, *sprG* antitoxin gene or *sprF*, *holin* antitoxin gene, *187-lysK* antitoxin gene, *yefM* antitoxin gene, *lysostaphin* antitoxin gene, or *mazE* antitoxin gene, *kpn1* antitoxin gene, *sma1* antitoxin gene, *relF* antitoxin gene, *rsaE* antitoxin gene, or *yoeB* antitoxin gene, respectively.

22. The synthetic microorganism of claim 21, wherein the antitoxin gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 273, 306, 307, 308, 309, 310, 311, 312, 314, 319, or 322, or a substantially identical nucleotide sequence.

23. The synthetic microorganism of claim 21 or 22, wherein the second promoter comprises or is derived from a gene selected from the group consisting of *clfB* (Clumping factor B), *sceD* (autolysin, exoprotein D), *walKR* (virulence regulator), *atlA* (Major autolysin), *oatA* (O-acetyltransferase A); phosphoribosylglycinamide formyltransferase gene, phosphoribosylaminoimidazole synthetase gene, amidophosphoribosyltransferase gene, phosphoribosylformylglycinamide synthase gene, phosphoribosylformylglycinamide synthase gene,

phosphoribosylaminoimidazole-succinocarboxamide gene, trehalose permease IIC gen, DeoR family transcriptional regulator gene, phosphofructokinase gene, PTS fructose transporter subunit IIC gene, galactose-6-phosphate isomerase gene, NarZ, NarH, NarT, alkylhydroperoxidase gene, hypothetical protein gene, DeoR trans factor gene, lysophospholipase gene, protein disaggregation chaperon gene, alkylhydroperoxidase gene, phosphofructokinase gene, *gyrB*, *sigB*, and *rho*.

24. The synthetic microorganism of claim 23, wherein the second promoter is a P_{clfB} (clumping factor B) and comprises a nucleotide sequence of SEQ ID NO: 117, 118, 129 or 130, or a substantially identical nucleotide sequence thereof.

25. The synthetic microorganism according to any one of claims 1 to 24, further comprising a molecular modification selected from the group consisting of a virulence block molecular modification, and nanofactory molecular modification.

26. The synthetic microorganism of claim 25, wherein the virulence block molecular modification prevents horizontal gene transfer of genetic material from the undesirable microorganism.

27. The synthetic microorganism of claim 25, wherein the nanofactory molecular modification comprises an insertion of a gene that encodes, a knock out of a gene that encodes, or a genetic modification of a gene that encodes a product selected from the group consisting of an enzyme, amino acid, metabolic intermediate, and a small molecule.

28. A composition comprising an effective amount of the synthetic microorganism of any one of claims 1 to 27, and a pharmaceutically acceptable carrier, diluent, emollient, binder, excipient, lubricant, sweetening agent, flavoring agent, wetting agent, preservative, buffer, or absorbent, or a combination thereof.

29. The pharmaceutical composition of claim 28, further comprising a nutrient, prebiotic, commensal, and/or probiotic bacterial species.

30. A single dose unit comprising the composition of claim 28 or 29.

31. The single dose unit of claim 30, comprising at least at least 10^5 , at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} CFU, or at least 10^{11} of the synthetic strain and a pharmaceutically acceptable carrier.

32. The dose unit of claim 31 formulated for topical administration.

33. The synthetic microorganism of any one of claims 1 to 27 or the composition of claim 28 or 29 for use in the manufacture of a medicament for eliminating and preventing the recurrence of a undesirable microorganism in a subject.

34. A method for eliminating and preventing the recurrence of a undesirable microorganism in a subject hosting a microbiome, comprising:

- a. decolonizing the host microbiome; and
- b. durably replacing the undesirable microorganism by administering to the subject a synthetic microorganism comprising at least one element imparting a non-native attribute, wherein the synthetic microorganism is capable of durably integrating to the host microbiome, and occupying the same niche in the host microbiome as the undesirable microorganism.

35. The method of claim 34, wherein the decolonizing is performed on at least one site in the subject to substantially reduce or eliminate the detectable presence of the undesirable microorganism from the at least one site.

36. The method of claim 35, wherein the detectable presence of the undesirable microorganism is determined by a method comprising a phenotypic method and/or a genotypic method, optionally wherein the phenotypic method is selected from the group consisting of biochemical reactions, serological reactions, susceptibility to anti-microbial agents, susceptibility to phages, susceptibility to bacteriocins, and/or profile of cell proteins, and optionally

wherein the genotypic method is selected from the group consisting of hybridization, plasmids profile, analysis of plasmid polymorphism, restriction enzymes digest, reaction and separation by Pulsed-Field Gel Electrophoresis (PFGE), ribotyping,

polymerase chain reaction (PCR) and its variants, Ligase Chain Reaction (LCR), and Transcription-based Amplification System (TAS).

37. The method of claim 35, wherein the niche is a dermal or mucosal environment that allows stable colonization of the undesirable microorganism at the at least one site.

38. The method of claim 37, wherein the ability to durably integrate to the host microbiome is determined by detectable presence of the synthetic microorganism at the at least one site for a period of at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

39. The method of claim 38, wherein the ability to durably replace the undesirable microorganism is determined by the absence of detectable presence of the undesirable microorganism at the at least one site for a period of at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

40. The method of claim 39, wherein the ability to occupy the same niche is determined by absence of co-colonization of the undesirable microorganism and the synthetic microorganism at the at least one site after the administering step,

optionally wherein the absence of co-colonization is determined at least one week, at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 26 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

41. The method of claim 34, wherein the at least one element imparting the non-native attribute is durably incorporated to the synthetic microorganism.

42. The method of claim 41, wherein the at least one element imparting the non-native attribute is durably incorporated to the host microbiome via the synthetic microorganism.

43. The method of claim 42, wherein the at least one element imparting the non-native attribute is selected from the group consisting of kill switch molecular modification, virulence block molecular modification, metabolic modification, and nano factory molecular modification.

44. The method of claim 43, wherein the molecular modification is integrated to a chromosome of the synthetic microorganism.

45. The method of claim 43, wherein the synthetic microorganism comprises a virulence block molecular modification that prevents horizontal gene transfer of genetic material from the undesirable microorganism.

46. The method of claim 43, wherein the synthetic microorganism comprises a kill switch molecular modification that reduces or prevents infectious growth of the synthetic microorganism under systemic conditions in the subject.

47. The method of claim 43, wherein the synthetic microorganism is derived from a target microorganism having the same genus and species as the undesirable microorganism.

48. The method of claim 43, wherein the synthetic microorganism is derived from a target microorganism that has the ability to biologically integrate with the decolonized host microbiome.

49. The method of claim 43, wherein the synthetic microorganism is derived from a target microorganism isolated from the host microbiome.

50. The method of claim 48 or 49, wherein the target microorganism is susceptible to at least one antimicrobial agent.

51. The method of claim 50, wherein the target microorganism is selected from a bacterial, fungal, or protozoal target microorganism.

52. The method of claim 51, wherein the target microorganism is a bacterial species capable of colonizing a dermal and/or mucosal niche and is a member of a genus selected from the group consisting of *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Pseudomonas*.

53. The method of claim 52, wherein the target microorganism is selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*, optionally wherein the target strain is a *Staphylococcus aureus* 502a strain or RN4220 strain.

54. The method of claim 46, wherein the synthetic microorganism kill switch molecular modification comprises a first cell death gene operably linked to a first regulatory region comprising a first inducible promoter.

55. The method of claim 54, wherein the first promoter is activated (induced) by a change in state in the microorganism environment in contradistinction to the normal physiological (niche) conditions at the at least one site in the subject.

56. The method of claim 55, wherein measurable average cell death of the synthetic microorganism occurs within at least a preset period of time following induction of the first promoter.

57. The method of claim 56, wherein the measurable average cell death occurs within at least a preset period of time selected from the group consisting of within at least 1, 5, 15, 30, 60, 90, 120, 180, 240, 300, or 360 min minutes following change of state.

58. The method of claim 57, wherein the measurable average cell death is at least a 50% cfu, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% cfu count reduction following the preset period of time.

59. The method of claim 55, wherein the change in state is selected from one or more of pH, temperature, osmotic pressure, osmolality, oxygen level, nutrient concentration, blood concentration, plasma concentration, serum concentration, metal concentration, chelated metal concentration, change in composition or concentration of one or more immune factors, mineral concentration, and electrolyte concentration.

60. The method of claim 59, wherein the change in state is a higher concentration of and/or change in composition of blood, serum, or plasma compared to normal physiological (niche) conditions at the at least one site in the subject.

61. The method of claim 60, wherein the first promoter is a blood, serum, plasma, and/or heme responsive promoter.

62. The method of any one of claims 55 to 61, wherein the first promoter is upregulated by at least 1.5 fold, at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold within a period of time selected from the group consisting of at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, and at least 360 min following the change of state.

63. The method of claim 62, wherein the first promoter is not induced, induced less than 1.5 fold, or is repressed in the absence of the change of state.

64. The method of claim 63, wherein the first promoter is induced at least 1.5, 2, 3, 4, 5 or at least 6 fold within a period of time in the presence of serum or blood.

65. The method of claim 64, wherein the first promoter is not induced, induced less than 1.5 fold, or repressed under the normal physiological (niche) conditions at the at least one site.

66. The method of claim 64, wherein the first promoter is not induced, induced less than 1.5 fold, or is repressed in the absence of blood, serum, plasma, or heme.

67. The method of any one of claim 54 to 66, wherein the synthetic microorganism is derived from a target microorganism that is a *Staphylococcus aureus* strain, and wherein the first promoter is derived from a gene selected from the group consisting of *isdA* (iron-regulated surface determinant protein A), *isdB* (iron-regulated surface determinant protein B), *isdG* (heme-degrading monooxygenase), *hlgA* (gamma-hemolysin component A), *hlgA1* (gamma-hemolysin), *hlgA2* (gamma-hemolysin), *hlgB* (gamma-hemolysin component B), *hrtAB* (heme-regulated transporter), *sbnC* (luc C family siderophore biosynthesis protein), *sbnD*, *sbnI*, *sbnE* (lucA/lucC family siderophore biosynthesis protein), *isdI*, *lrgA* (murein hydrolase regulator A), *lrgB* (murein hydrolase regulator B), *ear* (Ear protein), *fhuA* (ferrichrome transport ATP-binding protein fhuA), *fhuB* (ferrichrome transport permease), *hly* (phospholipase C), heme ABC transporter 2 gene, heme ABC transporter gene, *isd* ORF3, *sbnF*, alanine dehydrogenase gene, diaminopimelate decarboxylase gene, iron ABC transporter gene, threonine dehydratase gene, siderophore ABC transporter gene, SAM dep Metrans gene, *HarA*, *splF* (serine protease SplF), *splD* (serine protease SplD), *dps* (general stress protein 20U), SAUSA300_2617 (putative cobalt ABC transporter, ATP-binding protein), SAUSA300_2268 (sodium/bile acid symporter family protein), SAUSA300_2616 (cobalt family transport protein), *srtB* (Sortase B), *sbnA* (probable siderophore biosynthesis protein sbnA), *sbnB*, *sbnG*, *leuA* (2-isopropylmalate synthase amino acid biosynthetic enzyme), *sslA* (iron transport membrane protein), *sirA* (iron ABC transporter substrate-binding protein), *isdA* (heme transporter), and *spa* (Staphylococcal protein A).

68. The method of claim 67, wherein the first promoter is derived from or comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 114, 115, 119, 120, 121, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, and 163, or a substantially identical nucleotide sequence thereof.

69. The method of any one of claims 54 to 68, wherein the undesirable microorganism is a *Staphylococcus aureus* strain, and wherein the detectable presence

is measured by a method comprising obtaining a sample from the at least one site of the subject, contacting a chromogenic agar with the sample, incubating the contacted agar and counting the positive cfus of the bacterial species after a predetermined period of time.

70. The method of any one of claims 54 to 69, wherein the cell death gene is selected from a toxin gene selected from the group consisting of sprA1, sprA2, kpn1, sma1, sprG, relF, rsaE, yoeB, mazF, yefM, and lysostaphin toxin gene.

71. The method of claim 70, wherein the cell death gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 122, 124, 125, 126, 127, 128, 274, 275, 284, 286, 288, 290, 315, and 317, or a substantially identical nucleotide sequence.

72. The method of any one of claims 54 to 71, wherein the synthetic microorganism further comprises an expression clamp molecular modification comprising an antitoxin gene specific for the first cell death gene, wherein the antitoxin gene is operably linked to a second regulatory region comprising a second promoter which is active upon dermal or mucosal colonization or in TSB media, but is repressed at least 2 fold upon exposure to blood, serum or plasma after a period of time selected from the group consisting of the group consisting of at least 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, and at least 360 min.

73. The method of claim 72, wherein the antitoxin gene encodes an antisense RNA sequence capable of hybridizing with at least a portion of the first cell death gene.

74. The method of claim 73, wherein the antitoxin gene is selected from the group consisting of a sprA1 antitoxin gene, sprA2 antitoxin gene, sprG antitoxin gene or sprF, holin antitoxin gene, 187-lysK antitoxin gene, yefM antitoxin gene, lysostaphin antitoxin gene, or mazE antitoxin gene, kpn1 antitoxin gene, sma1 antitoxin gene, relF antitoxin gene, rsaE antitoxin gene, or yoeB antitoxin gene, respectively.

75. The method of claim 74, wherein the antitoxin gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 273, 306, 307, 308, 309, 310, 311, 312, 314, 319, or 322 or a substantially identical nucleotide sequence

76. The method of any one of claims 72 to 75, wherein the second promoter is derived from a gene selected from the group consisting of *clfB* (Clumping factor B), *sceD* (autolysin, exoprotein D), *walKR* (virulence regulator), *atlA* (Major autolysin), *oatA* (O-acetyltransferase A); phosphoribosylglycinamide formyltransferase gene, phosphoribosylaminoimidazole synthetase gene, amidophosphoribosyltransferase gene, phosphoribosylformylglycinamide synthase gene, phosphoribosylformylglycinamide synthase gene, phosphoribosylaminoimidazole-succinocarboxamide gene, trehalose permease IIC gen, DeoR family transcriptional regulator gene, phosphofructokinase gene, PTS fructose transporter subunit IIC gene, galactose-6-phosphate isomerase gene, *NarZ*, *NarH*, *NarT*, alkylhydroperoxidase gene, hypothetical protein gene, DeoR trans factor gene, lysophospholipase gene, protein disaggregation chaperon gene, alkylhydroperoxidase gene, phosphofructokinase gene, *gyrB*, *sigB*, and *rho*.

77. The method of claim 76, wherein the second promoter is a P_{clfB} (clumping factor B) and comprises a nucleotide sequence of SEQ ID NO: 117, 118, 129 or 130, or a substantially identical nucleotide sequence thereof.

78. The method of any one of claims 43 to 77, wherein the decolonizing step comprises topically administering a decolonizing agent to the at least one site in the subject to reduce or eliminate the presence of the undesirable microorganism from the at least one site.

79. The method of claim 78, wherein the decolonizing step comprises topical administration of the decolonizing agent, wherein no systemic antimicrobial agent is simultaneously administered.

80. The method of claim 78 or 79, wherein no systemic antimicrobial agent is administered within one week, two weeks, three weeks, one month, two months, three

months, six months, or one year of the first topical administration of the decolonizing agent.

81. The method of any one of claims 40 to 42, wherein the decolonizing agent is selected from the group consisting of a disinfectant, bacteriocide, antiseptic, astringent, and antimicrobial agent.

82. The method of claim 81, wherein the decolonizing agent is selected from the group consisting of alcohols (ethyl alcohol, isopropyl alcohol), aldehydes (glutaraldehyde, formaldehyde, formaldehyde-releasing agents (noxythiolin = oxymethylenethiourea, tauroline, hexamine, dantoin), o-phthalaldehyde), anilides (triclocarban = TCC = 3,4,4'-trichlorocarbanilide), biguanides (chlorhexidine, alexidine, polymeric biguanides (polyhexamethylene biguanides with MW > 3,000 g/mol, vantocil), diamidines (propamidine, propamidine isethionate, propamidine dihydrochloride, dibromopropamidine, dibromopropamidine isethionate), phenols (fenticlor, p-chloro-m-xlenol, chloroxylenol, hexachlorophene), bis-phenols (triclosan, hexachlorophene), chloroxylenol (PCMX), quaternary ammonium compounds (cetrimide, benzalkonium chloride, cetyl pyridinium chloride), silver compounds (silver sulfadiazine, silver nitrate), peroxy compounds (hydrogen peroxide, peracetic acid, benzoyl peroxide), iodine compounds (povidone-iodine, poloxamer-iodine, iodine), chlorine-releasing agents (sodium hypochlorite, hypochlorous acid, chlorine dioxide, sodium dichloroisocyanurate, chloramine-T), copper compounds (copper oxide), isotretinoin, sulfur compounds, botanical extracts (*Melaleuca* spp. (tea tree oil), (*Vaccinium* spp. (e.g., A-type proanthocyanidins), *Cassia fistula* Linn, *Baekea frutesdens* L., *Melia azedarach* L., *Muntingia calabura*, *Vitis vinifera* L., *Terminalia avicennioides* Guill & Perr., *Phyllanthus discoideus* Muel. Muel-Arg., *Ocimum gratissimum* Linn., *Acalypha wilkesiana* Muell-Arg., *Hypericum pruinatum* Boiss.&Bal., *Hypericum olimpicum* L. and *Hypericum sabrum* L., *Hamamelis virginiana* (witch hazel), Clove oil, *Eucalyptus* spp., *rosmarinus officinalis* spp. (rosemary), *thymus* spp. (thyme), *Lippia* spp. (oregano), lemongrass spp., *cinnamomum* spp., *geranium* spp., *lavendula* spp.), aminolevulonic acid, and topical antibiotic compounds (bacteriocins; mupirocin, bacitracin, neomycin, polymyxin B, gentamicin).

83. The method of claim 81 or 82, wherein the antimicrobial agent is selected from the group consisting of vancomycin, cefazolin, cephalothin, cephalexin, linezolid, daptomycin, clindamycin, lincomycin, mupirocin, bacitracin, neomycin, polymyxin B, gentamicin, prulifloxacin, ulifloxacin, fidaxomicin, minocyclin, metronidazole, metronidazole, sulfamethoxazole, ampicillin, trimethoprim, ofloxacin, norfloxacin, tinidazole, norfloxacin, ornidazole, levofloxacin, nalidixic acid, ceftriaxone, azithromycin, cefixime, ceftriaxone, cefalexin, ceftriaxone, rifaximin, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, gatifloxacin, gemifloxacin, prufloxacin, ulifloxacin, moxifloxacin, nystatin, amphotericin B, flucytosine, ketoconazole, posaconazole, clotrimazole, voriconazole, griseofulvin, miconazole nitrate, and fluconazole.

84. The method of any one of claims 78 to 83, wherein the decolonizing comprises topically administering the decolonizing agent at least one, two, three, four, five or six or more times prior to the replacing step.

85. The method of claim 84, wherein the decolonizing step comprises administering the decolonizing agent to the at least one host site in the subject from one to six or more times or two to four times at intervals of between 0.5 to 48 hours apart, and wherein the replacing step is performed after the final decolonizing step.

86. The method of claim 85, wherein the replacing step comprises initial topical administration of a composition comprising at least 10^5 , at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} CFU, or at least 10^{11} of the synthetic strain and a pharmaceutically acceptable carrier to the at least one host site in the subject.

87. The method of claim 86, wherein the initial replacing step is performed within 12 hours, 24 hours, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days or 7 days of the decolonizing step.

88. The method of claim 86 or 87, wherein the replacing step is repeated at intervals of no more than once every two weeks to six months following the final decolonizing step.

89. The method of claim 86 or 87, wherein the decolonizing step and the replacing step is repeated at intervals of no more than once every two weeks to six months.

90. The method of any one of claims 86 to 89, wherein the replacing comprises administering the synthetic microorganism to the at least one site at least one, two, three, four, five, six, seven, eight, nine, or ten times.

91. The method of claim 90, wherein the replacing comprises administering the synthetic microorganism to the at least one site no more than one, no more than two, no more than three times, or no more than four times per month.

92. The method of any one of claims 43 to 91, further comprising promoting colonization of the synthetic microorganism in the subject.

93. The method of claim 92, wherein the promoting colonization of the synthetic microorganism in the subject comprises administering to the subject a promoting agent, optionally where the promoting agent is a nutrient, prebiotic, commensal, stabilizing agent, humectant, and/or probiotic bacterial species.

94. The method of claim 93, wherein the promoting comprises administering from 10^6 to 10^{10} cfu, or 10^7 to 10^9 cfu of the probiotic bacterial species to the subject after the initial decolonizing step.

95. The method of claim 93, wherein the nutrient is selected from sodium chloride, lithium chloride, sodium glycerophosphate, phenylethanol, mannitol, tryptone, peptide, and yeast extract.

96. The method of claim 93, wherein the prebiotic is selected from the group consisting of short-chain fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid), glycerol, pectin-derived oligosaccharides from agricultural by-products, fructo-oligosaccharides (e.g., inulin-like prebiotics), galacto-oligosaccharides (e.g., raffinose), succinic acid, lactic acid, and mannan-oligosaccharides.

97. The method of claim 93, wherein the probiotic is selected from the group consisting of *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Streptococcus thermophiles*, and *Enterococcus faecalis*.

98. The method of claim 93, wherein the commensal is selected from the group consisting of *Acinetobacter johnsonii*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Corynebacterium acnes*, *Corynebacterium striatum*, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Cutibacterium acnes*, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Streptococcus pyogenes*, *Streptococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzae*, *Pseudomonas stutzeri*, *Pseudomonas putida*, and *Pseudomonas fluorescens*.

99. The method of any one of claims 43 to 98, wherein the undesirable microorganism is an antimicrobial agent-resistant microorganism.

100. The method of claim 99, wherein the antimicrobial agent-resistant microorganism is an antibiotic resistant bacteria.

101. The method of claim 99, wherein the antibiotic-resistant bacteria is a Gram-positive bacterial species selected from the group consisting of a *Streptococcus* spp., *Cutibacterium* spp., and a *Staphylococcus* spp.

102. The method of claim 101, wherein the *Streptococcus* spp. is selected from the group consisting of *Streptococcus pneumoniae*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*.

103. The method of claim 101, wherein the *Cutibacterium* spp. is selected from the group consisting of *Cutibacterium acnes* subsp. *acnes*, *Cutibacterium acnes* subsp. *defendens*, and *Cutibacterium acnes* subsp. *elongatum*.

104. The method of claim 101, wherein the *Staphylococcus* spp. is selected from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus*.

105. The method of claim 104, wherein the undesirable microorganism is a methicillin-resistant *Staphylococcus aureus* (MRSA) strain that contains a staphylococcal chromosome cassette (SCCmec types I-III), which encode one (SCCmec type I) or multiple antibiotic resistance genes (SCCmec type II and III), and/or produces a toxin.

106. The method of claim 105, wherein the toxin is selected from the group consisting of a Panton-Valentine leucocidin (PVL) toxin, toxic shock syndrome toxin-1 (TSST-1), staphylococcal alpha-hemolysin toxin, staphylococcal beta-hemolysin toxin, staphylococcal gamma-hemolysin toxin, staphylococcal delta-hemolysin toxin, enterotoxin A, enterotoxin B, enterotoxin C, enterotoxin D, enterotoxin E, and a coagulase toxin.

107. The method of any one of claims 43 to 106, wherein the subject does not exhibit recurrence of the undesirable microorganism as evidenced by swabbing the subject at the at least one site for at least two weeks, at least two weeks, at least four weeks, at least six weeks, at least eight weeks, at least ten weeks, at least 12 weeks, at least 16 weeks, at least 24 weeks, at least 30 weeks, at least 36 weeks, at least 42 weeks, or at least 52 weeks after the administering step.

108. A kit comprising in at least one container, the synthetic microorganism according to any one of claims 1 to 27, the composition according to any one of claims 28 or 29, or the single dose of any one of claims 30 to 32, and optionally at least a second container comprising a decolonizing agent, a sheet of instructions, at least a third container comprising a promoting agent, and/or an applicator.

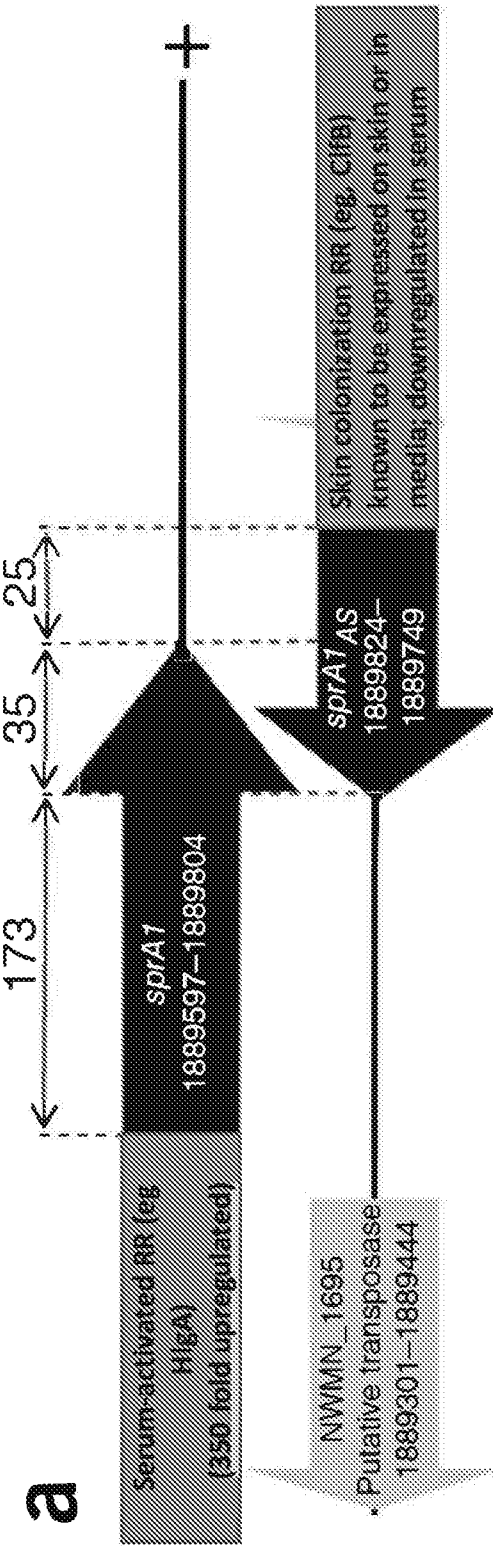


FIG. 1



FIG. 2

Table 4A. Recombinant Primers

SEQ ID NO:	Name	Sequence	Description
9	TKO1	GATGCGCATGCGAAACAGATTATCTCTATTC	LeuA PCR Amplification with SphI (upstream pr)
10	TKO2	GATGCGCATGCGAGATTATCTATTCAAAG	LeuA PCR Amplification with SphI (upstream pr-alternate)
11	TKO3	CATGAICTGCAGAGTAAATCCCCCGTAAATT	LeuA PCR Amplification with PstI (downstream pr)
12	TKO4	CACGTGAICTGCAGAGTAAATCCCCCGTAAAT	LeuA PCR Amplification with PstI (downstream pr-alternate)
13	TKO5	GACTACGAATTCAGGTGATGAAAAAATTTAGAA	upstream primer to amplify ClfB promoter with EcoRI
14	TKO6	GACTACGAATTCGTGAIGAAAAAATTTAGAACTT	backup to TKO5
15	TKO7	CTTAGCTGGATCCAAATAATTACTCCATTTCAA	downstream primer to amplify ClfB promoter with BamHI
16	TKO8	CTTAGCTGGATCCAAATAATTACTCCATTTCAAATTC	backup to TKO7
17	TKO9	GATGCGCATGCTCACAAACTATTGCGAAATC	upstream primer to amplify the HLGA RR; contains SphI
18	TKO10	GATGCGCATGCAAACTATTGCGAAATCCATTC	backup to TKO9
19	TKO11	CATGAICTGCAGATATATAATAATCCATTGT	downstream primer to amplify HLGA RR; contains PstI
20	TKO12	CATGAICTGCAGATATATAATAATCCATTGTAAAGCG	backup to TKO11
21	TKO13	GTGTTACGATAGCAAAATGCA	First sense primer for sequencing constructs containing pCAD promoter

FIG. 3A

Table 4A. Recombinant Primers (cont.)

SEQ ID NO:	Name	Sequence	Description
22	TKO14	TTATTGGCTAAGTAGACGCA	second sense sequencing primer anneals roughly in the middle of the SprA1 gene
23	TKO15	CACAATGTTCTTTCCTGCGTT	primer to anneal just upstream of the serum responsive RRs for LEUA and HLGA. Anneals in the PCN51 vector about 75 nt upstream of the SphI site
24	TKO16	ACGCGGCCTTTTACGGTTC	backup in case TKO15 produces low quality reads
25	TKO17	GAATGGGACTTGTAACGTC	primer to anneal near the downstream one third of the LEUA promoter/RR
26	TKO18	GAATGGGACTTGTAACG	backup to TKO17
27	TKO19	ATAACGCCCTGCGACCAATA	primer to anneal near the downstream one third of the HLGA promoter/RR
28	TKO20	GCGACCAATAAATCTTTTAA	Backup to TKO19 in case it produces low quality reads
29	TKO21	TTGAATAGATAATCTGTTTCGCATGCAGCGGCCGCCAGCT	pTK1 vector with leuA pro homology R
30	TKO22	AATTACGGGGGAATTTACTCTGCAGGGTACCGCAGAGAG	pTK1 vector with leuA pro homology F
31	TKO23	AGCTGGCGGCCGCTGCAATGCCGAAACAGATTACTATTCAAAGTTAATTG	leuA insert with pTK1 homology F
32	TKO24	CCTCTGCGGTACCCCTGCAGAGTAAATTCCTCCGTAATTTTAAATG	leuA insert with pTK1 homology R
33	TKO25	CATTAAAAATTACGGGGGAATTTACTCTGCAGATGAGCAGGGAATGA	pTK9 vector with leuA pro homology F

FIG. 3B

Table 4A. Recombinant Primers (cont.)

SEQ ID NO:	Name	Sequence	Description
34	TKO26	CAATTAACTTTGAAATAGATAATCTGTTTCGCAIGCAGCGGCCCGCAGCT	pTK9 vector with leuA pro homology R
35	TKO27	CATTAAAATTTACGGGGGAATTTACTCTGCAGATGGTAGAGATAGC	pTK12 vector with leuA pro homology F
36	TKO28	GCTATCTCTACCACTCTGCAGAGTAAATTCCCCGTAAATTTTAATG	leuA insert with pTK12 homology R
37	TKO29	gaatccatctgtgtcaatcatTATAACCCCTCTTTAATTGGTTATATG	pTKvector R with kanR homology (remove ermR)
38	TKO30	ccttcttgacgaghtctctgpaGTTAAGGGAIGCATAAACTGCA	pTKvector F with kanR homology (remove ermR)
39	TKO31	CATATAACCAAAATTAAAGAGGGTTATAaigattgaacaagatggattgc	pCASSA kanR F with pTK homology (add kanR)
40	TKO32	TGCAGTTTATGCATCCCTTAACicagaagaacigcicaagaagg	pCASSA kanR R with pTK homology (add kanR)
41	TKO33	GAATGGGACTTGTAAACGTCCC	leuA colony screen PCR F
42	TKO34	GGGACGTTTACAAGTCCCATTG	leuA colony screen PCR R

FIG. 3C

Table 4B. CRISPR Primers

SEQ ID NO:	BPC No.	Name	Sequence	Purpose
43	1	BPC - T1.1 - FOR	gaaaGGAGTAATATCGATGGAGTA	gRNA insertion for pCasSA
44	2	BPC - T1.1 - REV	caaaTACTCCATCGATATTACTCC	gRNA insertion for pCasSA
45	3	BPC - T1.2 - FOR	gaaaGGAGAGGATGATGATTATAA	gRNA insertion for pCasSA
46	4	BPC - T1.2 - REV	caaaTTATAATCATCATCTCTCTCC	gRNA insertion for pCasSA
47	5	BPC - T1.3 - FOR	gaaaGGGAGAGGATGATGATTATA	gRNA insertion for pCasSA
48	6	BPC - T1.3 - REV	caaaTATAATCATCATCTCTCTCCC	gRNA insertion for pCasSA
49	7	BPC - T1.4 - FOR	gaaaGGGCTCTAATGTTATTGCTTA	gRNA insertion for pCasSA
50	8	BPC - T1.4 - REV	caaaTAAGCAATAACATTAGACCC	gRNA insertion for pCasSA
51	9	BPC - T1.5 - FOR	gaaaGGAGAGGATGATGATTATA	gRNA insertion for pCasSA
52	10	BPC - T1.5 - REV	caaaTATAATCATCATCTCTCTCC	gRNA insertion for pCasSA
53	11	BPC - T1.6 - FOR	gaaaGGTAGTAGTATGAGTAATATCGA	gRNA insertion for pCasSA
54	12	BPC - T1.6 - REV	caaaTCGATATTACTCATACTACC	gRNA insertion for pCasSA
55	13	BPC - T1.7 - FOR	gaaaGGAATTATATAAATATAAAG	gRNA insertion for pCasSA
56	14	BPC - T1.7 - REV	caaaCTTTATATTTATATAAATTCC	gRNA insertion for pCasSA
57	15	BPC - T1.8 - FOR	gaaaGGCTACCTCCATATTTTCTA	gRNA insertion for pCasSA
58	16	BPC - T1.8 - REV	caaaTAGAAAAATATGGAGGTAGCC	gRNA insertion for pCasSA

FIG. 4A

Table 4B. CRISPR Primers (cont.)

SEQ ID NO:	BPC No.	Name	Sequence	Purpose
59	17	BPC - T1.9 - FOR	gaaaGGATAGAACTGTATTAGACT	gRNA insertion for pCasSA
60	18	BPC - T1.9 - REV	caaaAGTCTAATACAGTCTATCC	gRNA insertion for pCasSA
61	19	BPC - T1.10 - FOR	gaaaGGTGTCTAATGTTATTGCTT	gRNA insertion for pCasSA
62	20	BPC - T1.10 - REV	caaaAAGCAATAACATTAGACACC	gRNA insertion for pCasSA
63	21	BPC - gRNA - FOR	TGTTCTTTCCCTGCGTTGTCG	sequencing primers for gRNA insertion into the pCasSA vector
64	22	BPC - gRNA - REV	TCGCATTGACGTTAATACCTACAT	sequencing primers for gRNA insertion into the pCasSA vector
65	23	BPC - T1.1.2 - REV	aaacTACTCCATCGATATTACTCC	Correct reverse primers for gRNA
66	24	BPC - T1.2.2 - REV	aaacTTATAATCATCATCCTCTCTCC	Correct reverse primers for gRNA
67	25	BPC - T1.3.2 - REV	aaacTATAATCATCATCCTCTCTCC	Correct reverse primers for gRNA
68	26	BPC - T1.4.2 - REV	aaacTAAGCAATAACATTAGACCC	Correct reverse primers for gRNA
69	27	BPC - T1.5.2 - REV	aaacTATAATCAATCATCCTCTCTCC	Correct reverse primers for gRNA
70	28	BPC - T1.6.2 - REV	aaacTCGATATTACTCATACTACC	Correct reverse primers for gRNA
71	29	BPC - T1.7.2 - REV	aaacCTTTAATATTATATAAATTC	Correct reverse primers for gRNA
72	30	BPC - T1.8.2 - REV	aaacTAGAAAATATGGAGGTAGCC	Correct reverse primers for gRNA

FIG. 4B

Table 4B. CRISPR Primers (cont.)

SEQ ID NO:	BPC No.	Name	Sequence	Purpose
73	31	BPC - T1.9.2 - REV	aaacAGTCTAATACAGTTCTATCC	Correct reverse primers for gRNA
74	32	BPC - T1.10.2 - REV	aaacAAGCAATAACATTAGACACC	Correct reverse primers for gRNA
75	33	BPC - pCN51-1 - FOR	TTTGCTGGCCCTTTTGCTCAC	Primers to check insertion into pCN51
76	34	BPC - pCN51-1 - REV	TGCTTTTTCGATTGATGAACACCT	Primers to check insertion into pCN51
77	35	BPC - pCN51-2 - FOR	CGGCCTTTTACGGTTCTCTG	Primers to check insertion into pCN51
78	36	BPC - pCN51-2 - REV	ACGTTCCTTTTTCGATTGATGAAC	Primers to check insertion into pCN51
79	37	BPC - mChr-1 - FOR	ca ggtgacCTGCAGTCACATGGTGAGCAAGGGC	mCherry mRNA with PstI
80	38	BPC - mChr-1 - REV	gact acGAATTCAAAACACTGATTTCGTTGACCCG	mCherry mRNA with EcoRI
81	39	BPC - mChr-2 - REV	ccttagctGGATCCAAAACACTGATTTCGTTGACCCG	mCherry mRNA with BamHI alternate to #39
82	40	BPC - pCN51-hdr - REV	ccttagctCCCGGGTGCTTTTTCGATTGATGAACACCT	34BPC-REV with XmaI, use with TKO15 to add homologous arms
83	41	BPC - 2a - FOR	CGCCAAACGTTTCGTCAGTT	502a Target 1 genomic incorp check
84	42	BPC - 2a - REV	TTCAAGCGGTGACAAAGCAGC	502a Target 1 genomic incorp check
85	43	BPC - 2a - FOR	TGCGCAATGGCCAAAAGAT	502a Target 1 genomic incorp check
86	44	BPC - 2a - REV	CGTGCTAACATCCGCTTCAA	502a Target 1 genomic incorp check

FIG. 4C

Table 4B. CRISPR Primers (cont.)

SEQ ID	BPC No.	Name	Sequence	Purpose
87	45	BPC - mChr-1 - REV	AAAACTGATTTCGGTTGACCCG	mCherry
88	46	BPC - mChr-1 - FOR	caagtgatCTGCAGTCACATGGTTTCTAAAGGT	mCherry with PstI (codon opt seq in pJ204; 300995)
89	47	BPC - pJ204-1 - FOR	ACGTTGCTTTTTCGATTGATGA	Checking for insertion btwn HAs in pJ204
90	48	BPC - pJ204-1 - REV	TCCCCATGGGAGAGTAGGG	Checking for insertion btwn HAs in pJ204
91	49	BPC - pJ204-2 - FOR	GAATATTTAAGGGCGCTGTCAC	Checking for insertion btwn HAs in pJ204
92	50	BPC - pJ204-2 - REV	TATGGGGTGTCGCCCTTT	Checking for insertion btwn HAs in pJ204
93	51	BPC - mChr-1 - FOR	TCACATGGTTTCTAAAGGT	mCherry (codon opt seq in pJ204; 300995)
94	52	BP - repF-1 - F	CATGCTGCAGAACGGATTG	Checking for repF removal
95	53	BP - repF-1 - R	GCGCGGGAATAATGATGCTAA	Checking for repF removal
96	54	BP - repF-2 - F	AGTGACTGATGGCTGGTTG	Checking for repF removal
97	55	BP - repF-2 - R	TATGTCCTTTTGCACGTCGG	Checking for repF removal
98	56	BP - srtA - F	gaaaCAAACAAATATGCTGCCACT	srtA CRISPR targeting from Dong et al.
99	57	BP - srtA - R	aaacAGTGGCAGCATAATTGTTTG	srtA CRISPR targeting
100	58	BP - hla - F	gaaaGCTTCCAATATCTGTAGTAC	hla CRISPR targeting from Dong et al.
101	59	BP - hla - R	aaacGTACTACAGATATTGGAAGC	hla CRISPR targeting
102	60	BP - coa - F	gaaaGCCATTTTTTAAATCTGTACG	coa CRISPR targeting from Dong et al.
103	61	BP - coa - R	aaacCGTACAGATTTTAAAAAATGGC	coa CRISPR targeting

FIG. 4D

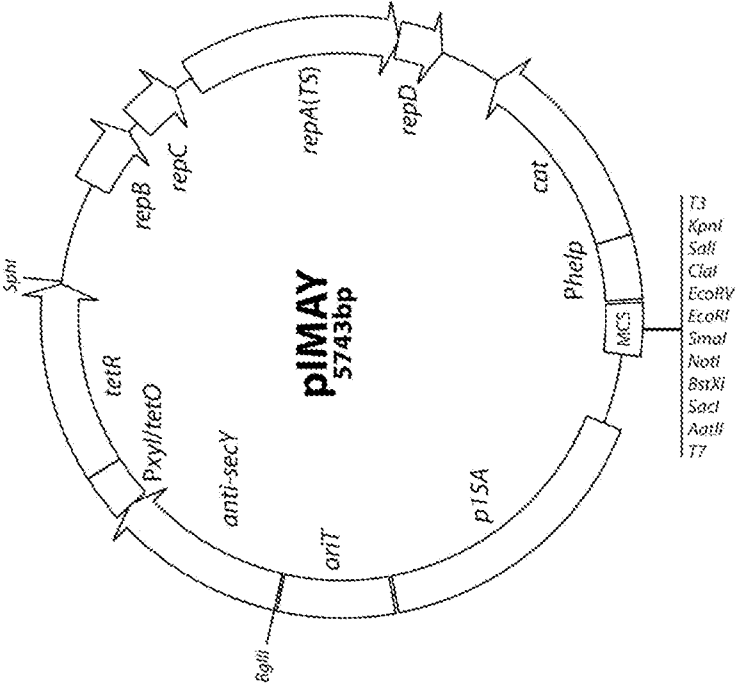


FIG. 5B

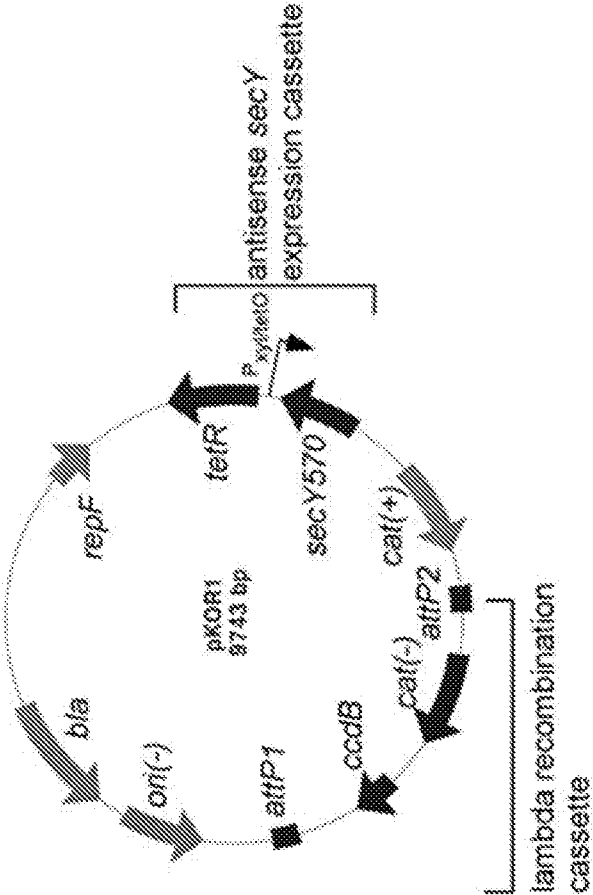


FIG. 5A

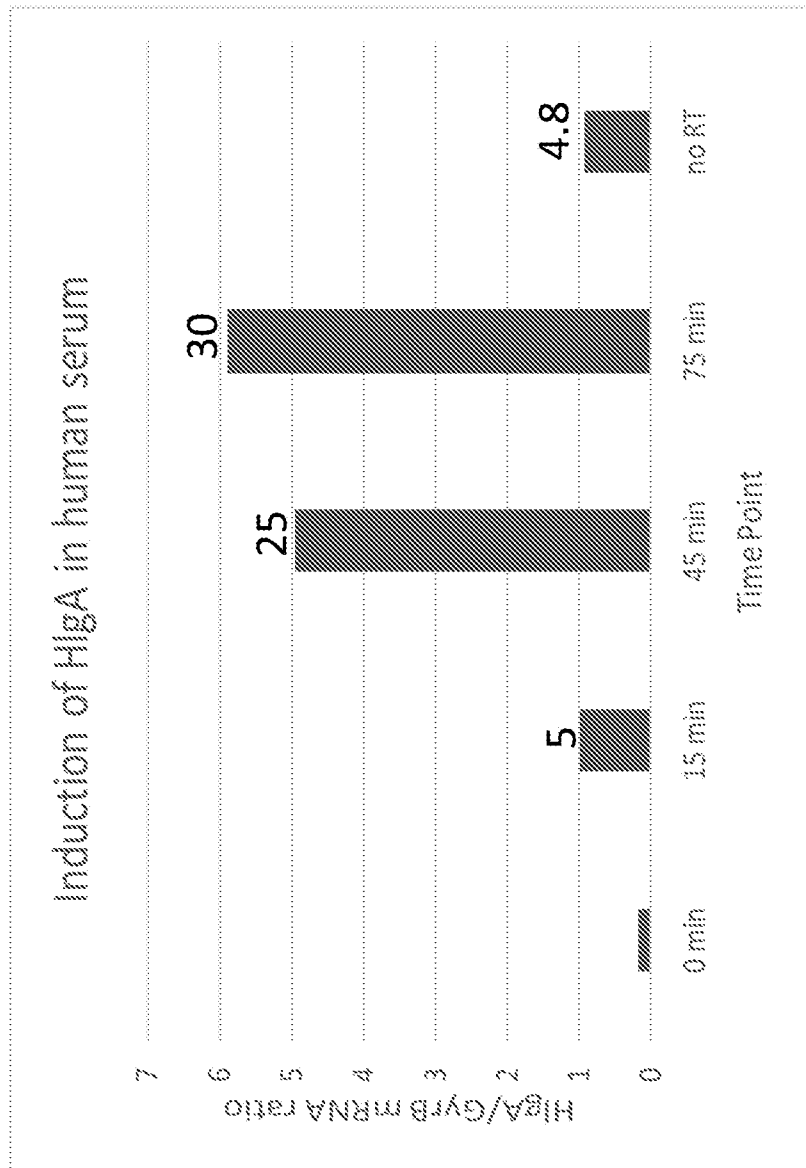


FIG. 6

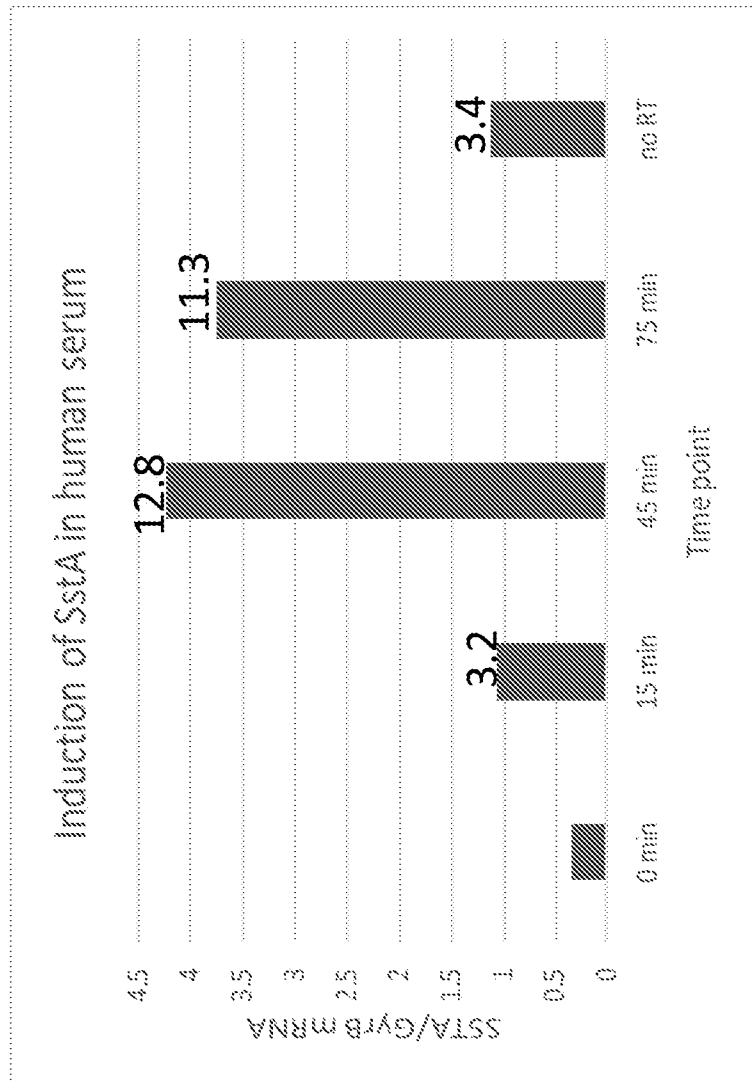


FIG. 7



[illegible]

FIG. 9

Cassette for integration via CRISPR

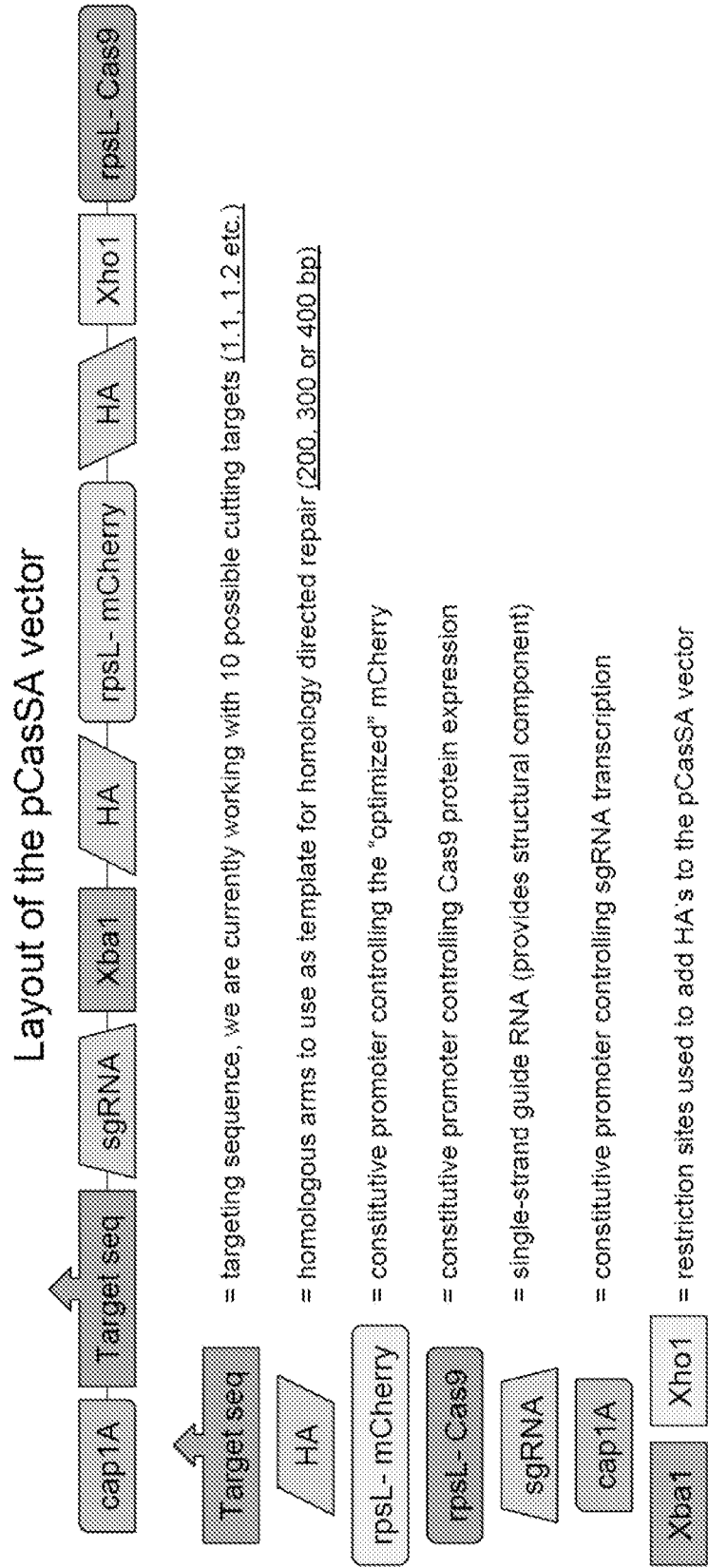


FIG. 10

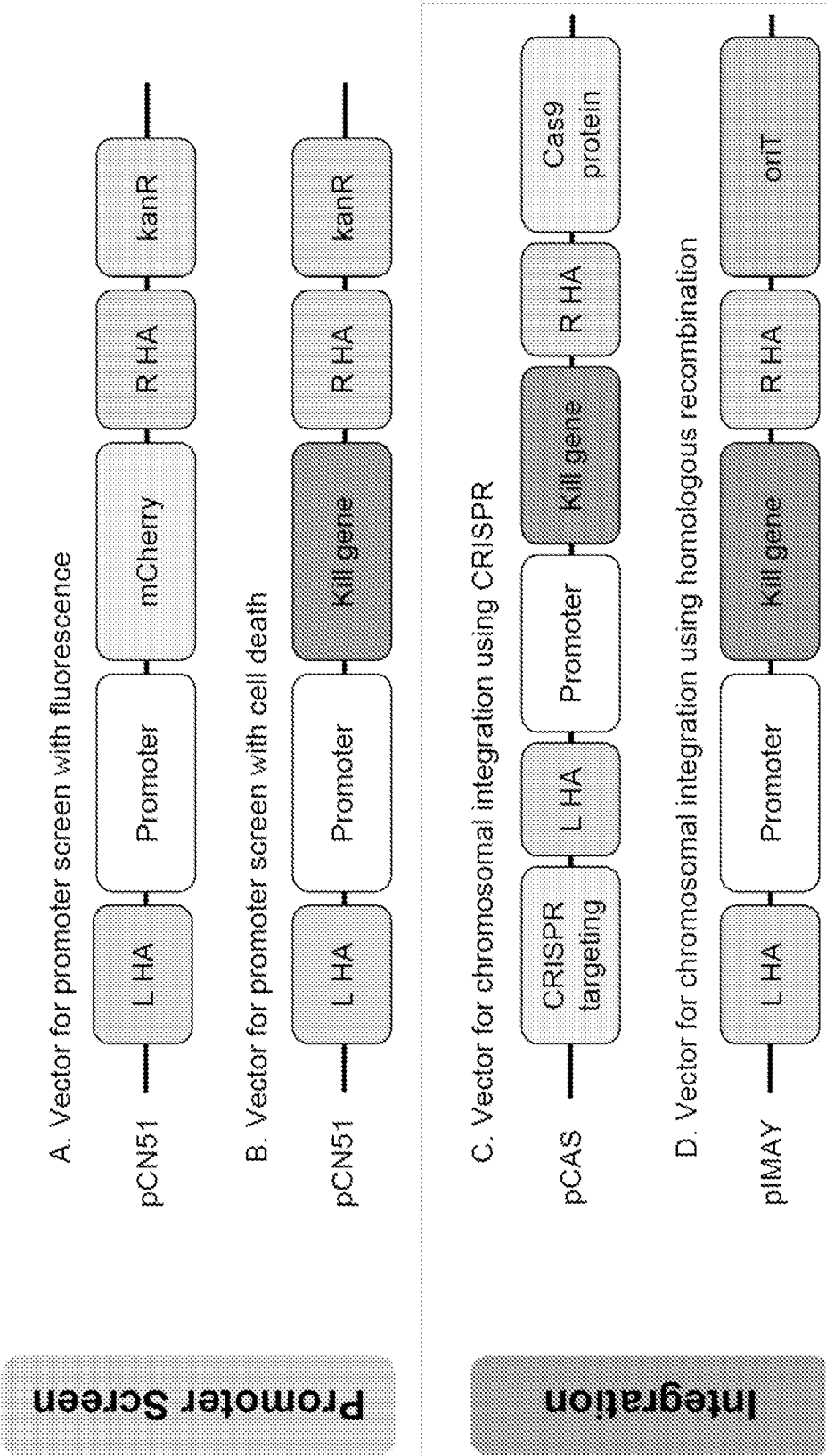


FIG. 11

1 gcatgcgttt tagcgtttat ttccgtttagt tatcggcata atcgttaaaa caggcgttat
 61 cgtagcgttaa aagcccttga gctagcgttg gctttgcagc gaagatgttg totgttagat
 121 tatgaagcc gatgactgaa tgaataata agcgcagcgc ccttctattt cggttggagg
 181 aggtcaagg gagtatgag gaatgaatt ccttcattgg ttgtatttta aaattgctt
 241 gcaattttgc ctagcgttag cgtggaaaaa aaatttgaa ttgtgaaaaa
 301 aatgggggga aggaagcga attttgcttc cgtactacga cccccatta agtcccgagt
 361 gccaattttt gtgccaaaaa cgtctatccc caactggctc aagggtttta ggggtttttc
 421 aatcgccaac gaatcgccaa cgttttgcgc aacgtttttt ataatctat atttaagtag
 481 ctttattgtt gtttttatga ttacaagtgc atacactaac ttataaaat tattgattg
 541 gagtttttta aatgtgtgatt tcagaatcga aaaaaagagt tatgatttct ctgacaaaag
 601 agcaagataa aaatttaaca gatabggcga acaaaaaagg ttttcaaaa tctggggttg
 661 cggcgttagc tatagaagaa tatgcaagaa aggaatcaga acaaaaaaaa taagcgaaaag
 721 ctgcggtttt tagaaggata cgagtttttc ctacttggtt ttgataaggt aattatatca
 781 tggctattaa aaatactaaa gctagaaatt ttggattttt attatatcct gactcaattc
 841 ctaatgattg gaaagaaaaa tttagagagt tggcgtatc tatggctgtc agtcccttac
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 961 gaaagcacta taaaaaacca cactatcag ttatatatat tgcacgaat cctgtaacaa
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 1261 atatagtggg tgactataat ttggttaata caaaagattt aatggctttt attcgctta
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 1381 ctagcgcct tagattatgg tttaggggca attatcagtg tggatataga gcaagttatg
 1441 caaaggttct tgatgctgaa acgggggaaa taaatgaca acaaaagaaa aagagttatt
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 1561 cagagaaatg gaagttgaat taagtacaac aatagattta ttgagaggag ggattattga
 1621 ataaataaaa gccccctgac gaaagtcgaa gggggttttt attttggttt gatgttgcga
 1681 ttaatagcaa tacattctat aatagaaggt atggaggatg ttatatatag agacagaatt
 1741 atgatgatca tatgtcaact aacggggcag gttagtgaac ttagaaaacc gactgtaaaa
 1801 agtcagtcg gcattatctc atattataa agccagtcac taggcoctatc tgacaattcc
 1861 tgaatagagt tcataaaca tcttgcatga taaccatcac aaacagaatg atgtacctgt
 1921 aaagatagcg gtaaatatat tgaattacct ttattaatga attttccctg tgtaataatg
 1981 ggtagaaggt aattactatt attattgata ttttaagttaa acccagtaaa tgaagtccat
 2041 ggaataatag aaagagaaa agcattttca ggtataggtg ttttgggaaa caatttccc
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 2221 ggtcttaact tatoccaata acctaaactc cgtcgtctat tgtaaccagt tctaaagct
 2281 gtatttgagt ttatcacct tgtcactaag aaataaaaa cagggtaaaa ttatatcct
 2341 tcttgtttta tgtttcggta taaaacacta atatcaattt ctgtggttat actaaagtc

FIG. 12A (SEQ ID NO: 131 cont.)

2401 gtttggttggt tcaataaatg attaaatatac tttttttctt tccaattgtc taaatcaatt
 2461 ttattaaagt tcatgggttt cactctcttt ctacattttt taacctaaata atgccaataa
 2521 ccgtttgcca cccctctctt tgataatat tgataatat aatattggcg aaattcgctt ctaaatga
 2581 aacgcaatat tatagtcttg ctttatggc cgtatgtgat tataccagcg cctcactac
 2641 atgtcaagaa taaactgcca aagcataatg aagcataatg ggataattaa cctcaactaa agggaacaaa
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 2761 tgcagcccg cggatccact agttctagag cggccgccac cgcggtggag ctccaattcg
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 2881 attatttat totgcaagt gatcttcctg cacttgatt tattoggcg aaagtgcgtc
 2941 ggggtgatgt gccaaactac tgatttagtg tatgatgtg ttttgaggt gctccagtgg
 3001 cttotgttcc taccagctgt cctcctgtt cagctactga cggggtgtg cgtaacggca
 3061 aaagcaccgc cggacatcag cgttagcggg gtgtatactg gttactatg ttggcactga
 3121 tgagggtgtc agtgaagtgc ttcattgtggc aggagaaaaa aggtgcacc ggtgcgtcag
 3181 cagaatatgt gatacaggat atattcgtc tctcgtcctc ctgactcgt acgtcgggtc
 3241 gttogactgc ggcgagcga aatggcttac gaacggggcg gagatttctt ggaagatgcc
 3301 aggaagatac ttaacaggga agtgagagg cgcgggcaaa gccgtttttc cataggctcc
 3361 gccccctga caagcatcac gaaatctgac gctcaaatca gtgtggcga aaccgacag
 3421 gactataaag ataccaggcg tttccctcgt cgtgcgtctt cctgttccctg
 3481 cctttcgtgt taccggtgtc attcgcgtgt tatggccggt tttgtctcat tccagcctg
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 3781 gttacctcgg ttcaaaaggt tggtagctca gagaaccttc gaaaaaccgc cctgaagggc
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 4021 acgccggcgg tagccggcca gcctgcaga gcaggattcc cgttgagcac cgcaggtgc
 4081 gaataaggga cagtgaagaa ggaacaccgc ctcgcggttg ggcctacttc acctatctg
 4141 cccggctgac gccgttggat acaccaagga aagttctcac gaacctttg gcaaatctc
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 4381 tgattcaaac ccttgtgaac tttcttagaa caaaagaggt tctgaacaa atttcttca
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 4561 gtggcggagc ctggaacga tttctctt ttgcaatggg tattgtaccc tacatcactg
 4621 catcaatcgt aatgcaatta ttacaatgg atattgtccc taaattctca gaatgggcaa
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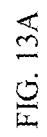
FIG. 12B (SEQ ID NO: 131 cont.)

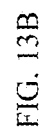
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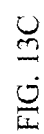
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4921 ctattatcat attcccatca agcttatttt aattatactc tatcaatgat agagtgtcaa
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5161 caaccgtaa actcgccag aagctagggtg tagagcagcc tacattgtat tggcatgtaa
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5401 cagaaaaaca gtatgaaact ctgaaaatc aattagcctt ttatgccc caaggttttt
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FIG. 12C (SEQ ID NO: 131 cont.)







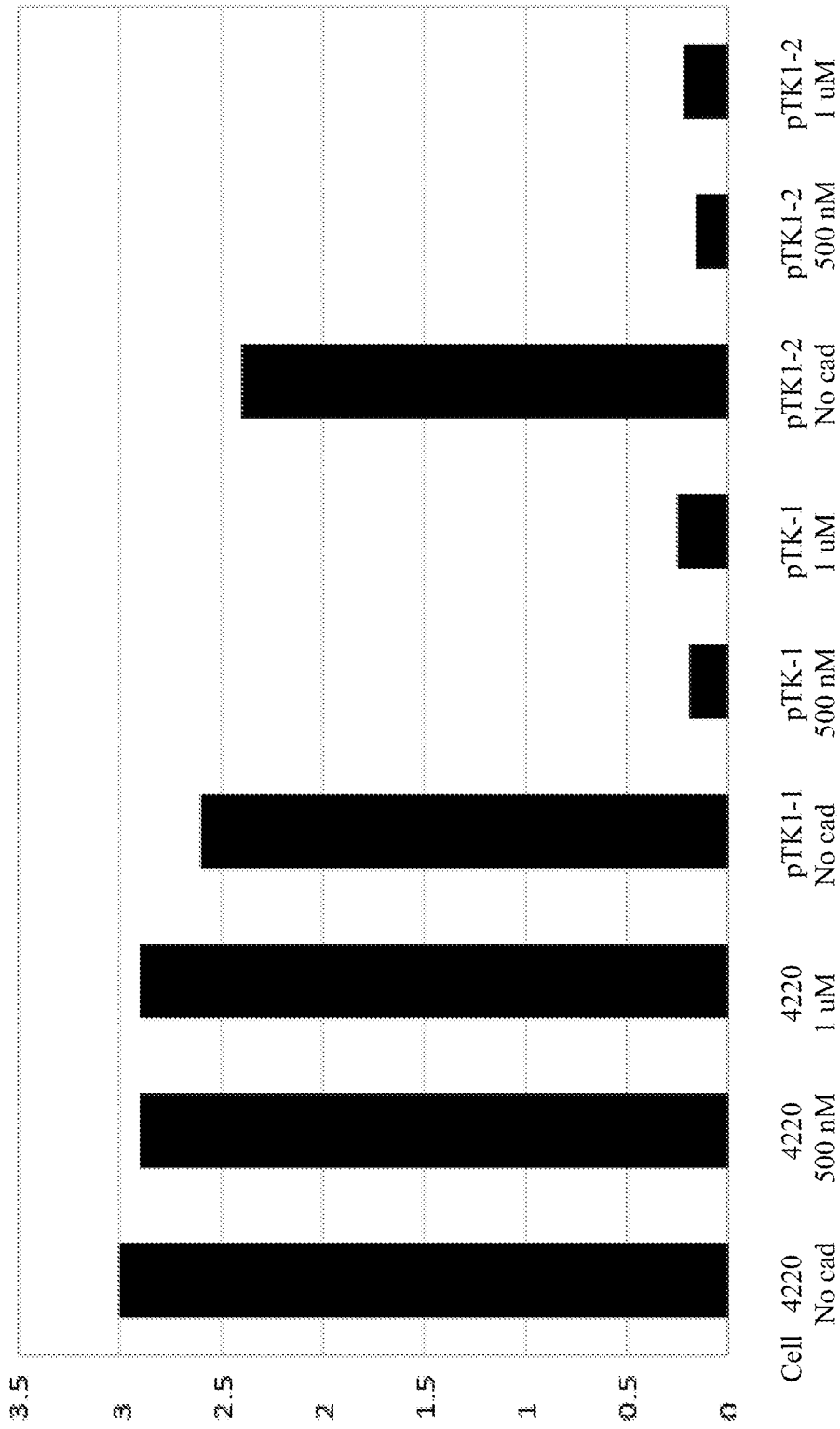


FIG. 14

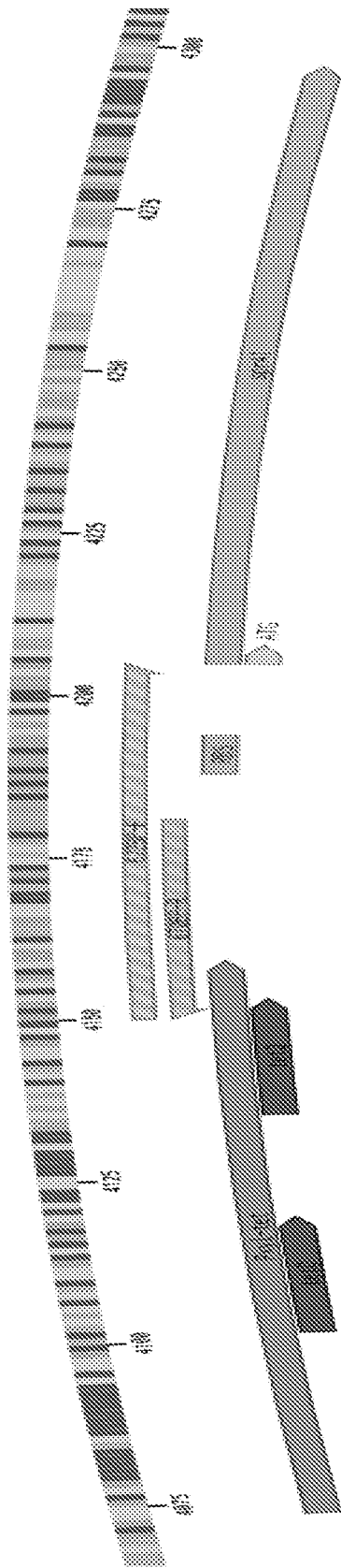


FIG. 15A

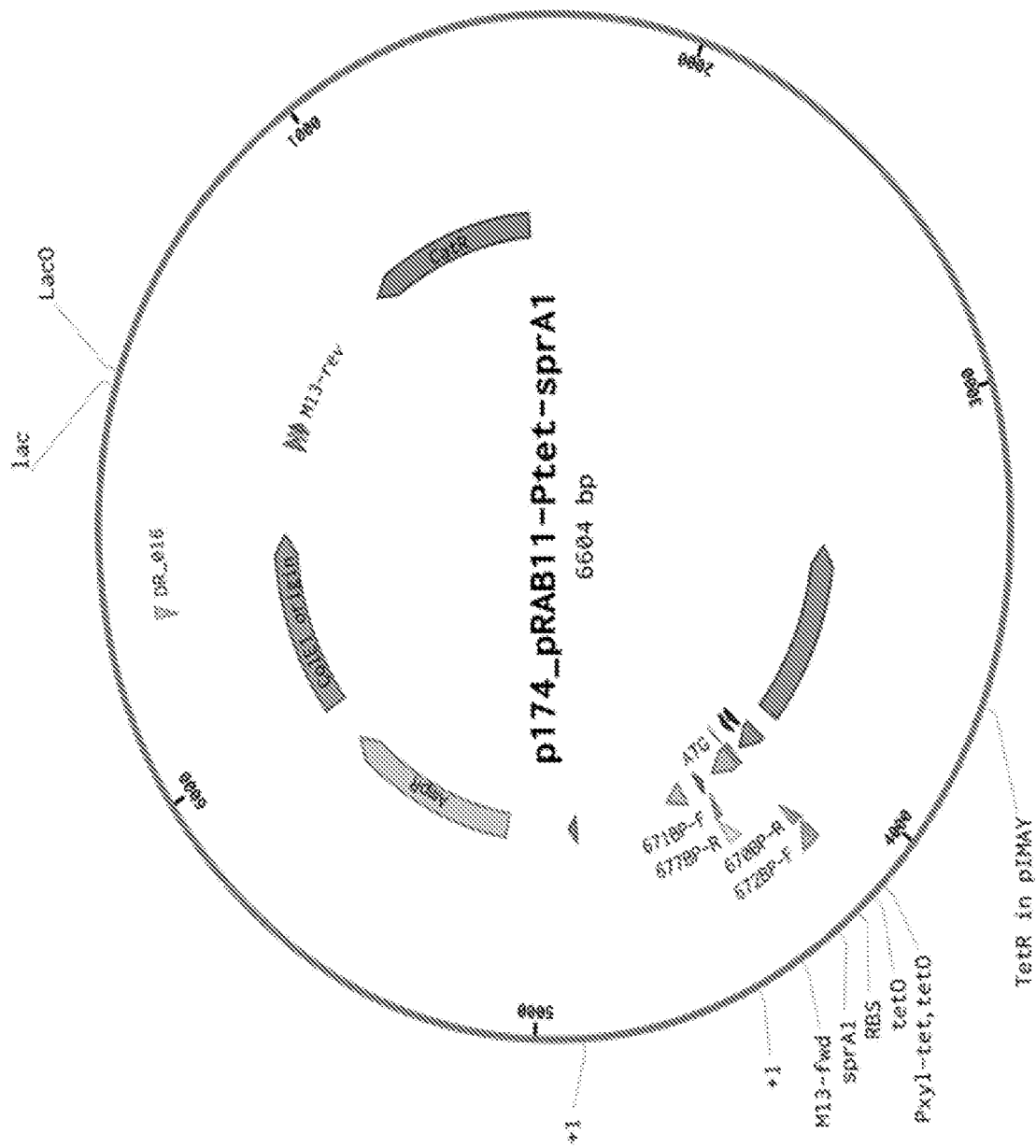


FIG. 15B



FIG. 15C

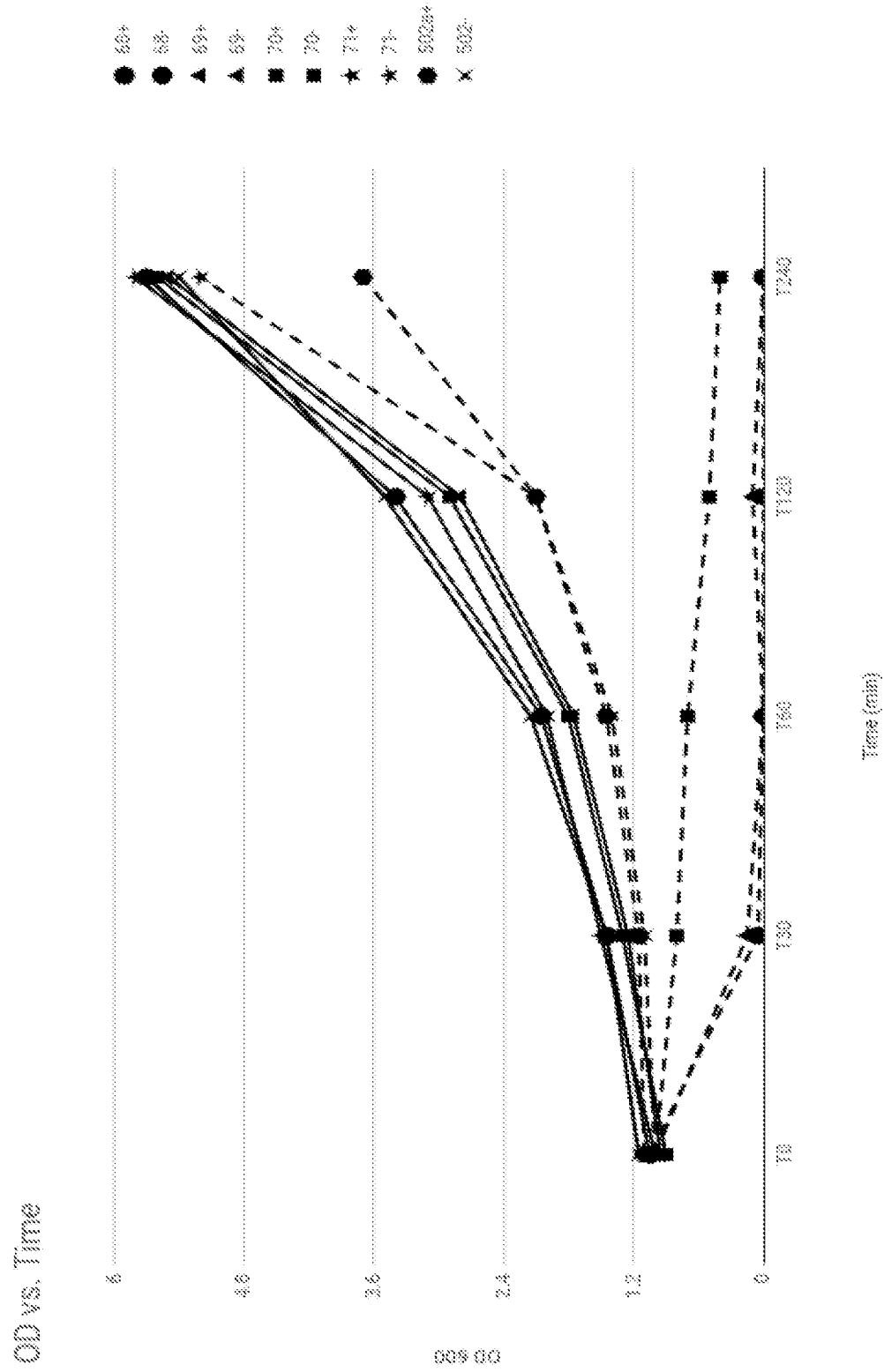


FIG. 16

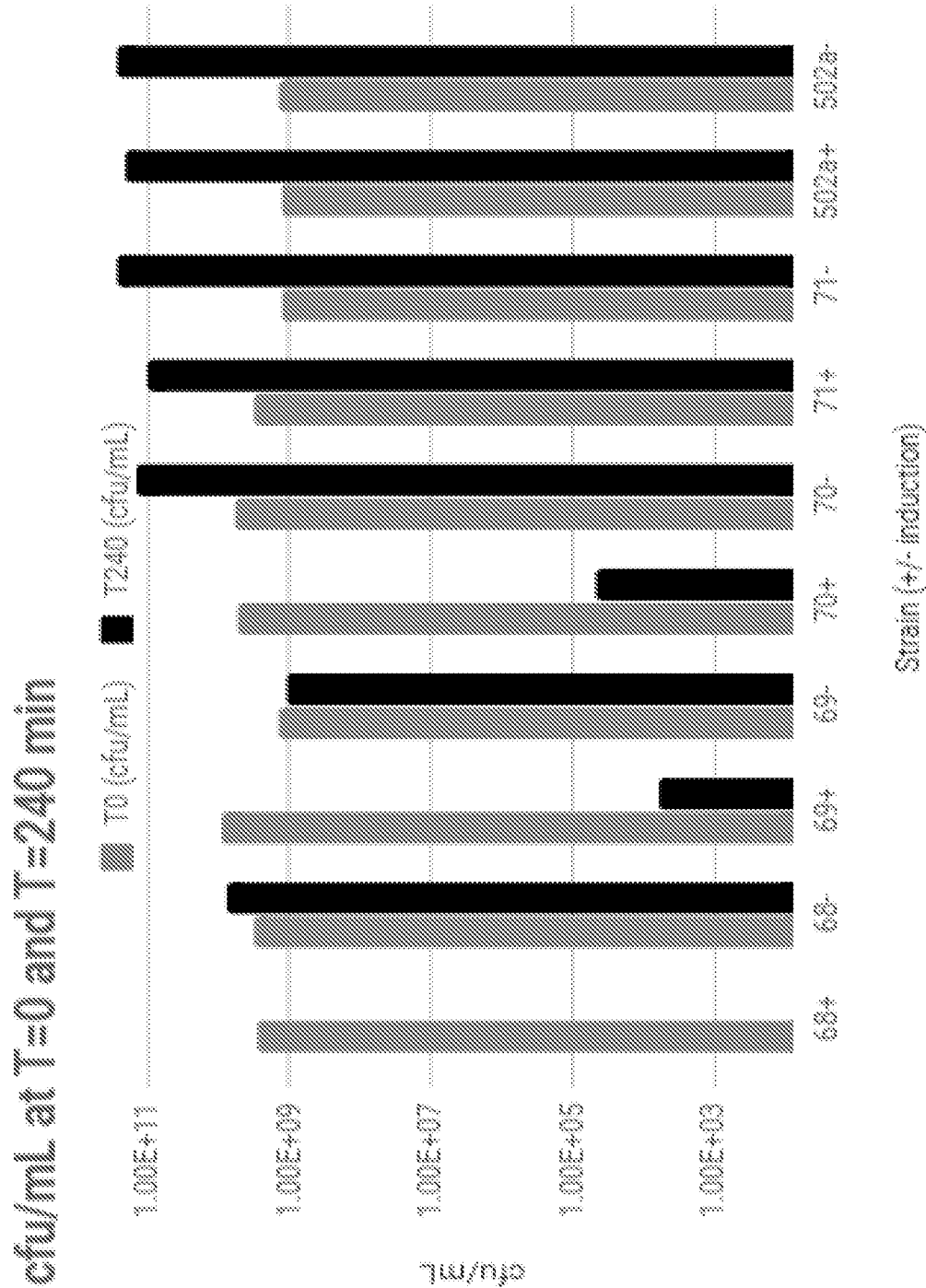


FIG. 17

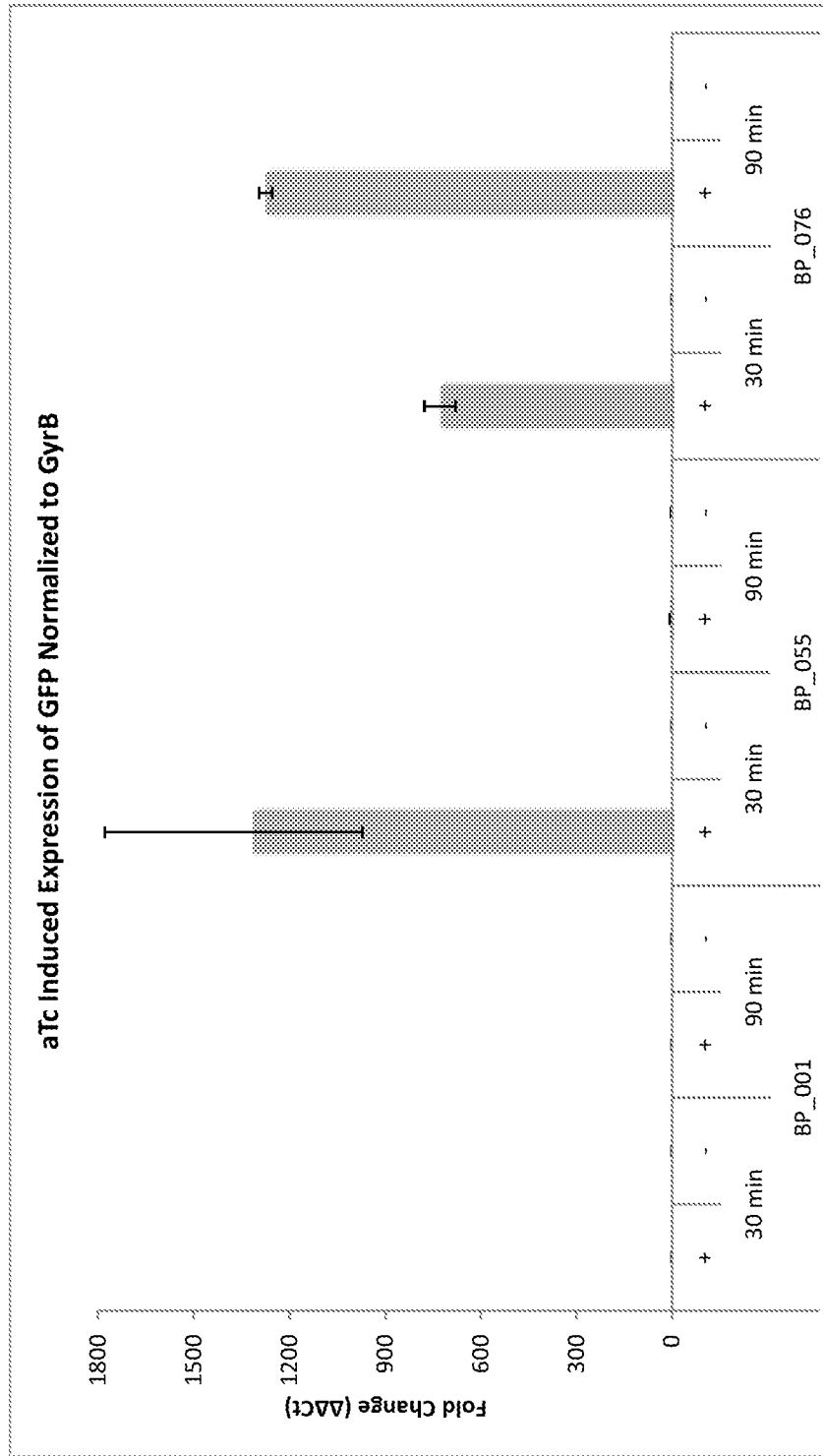


FIG. 18

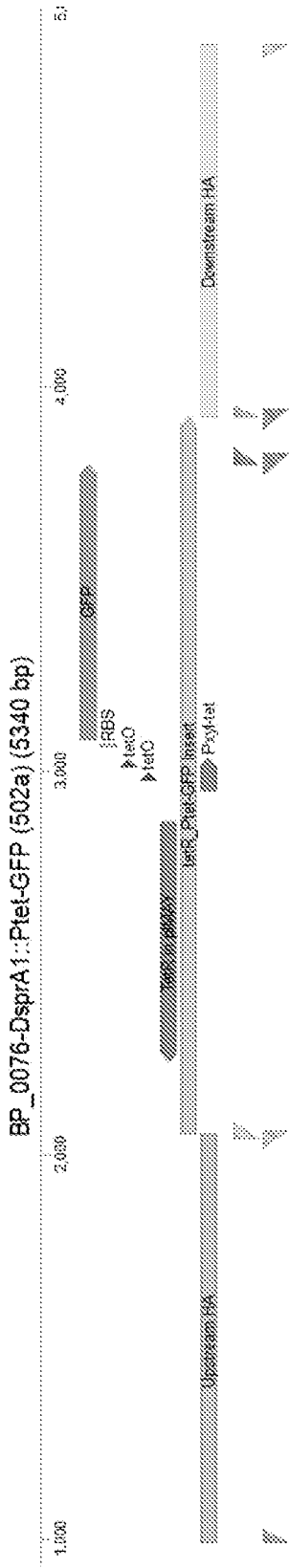


FIG. 19

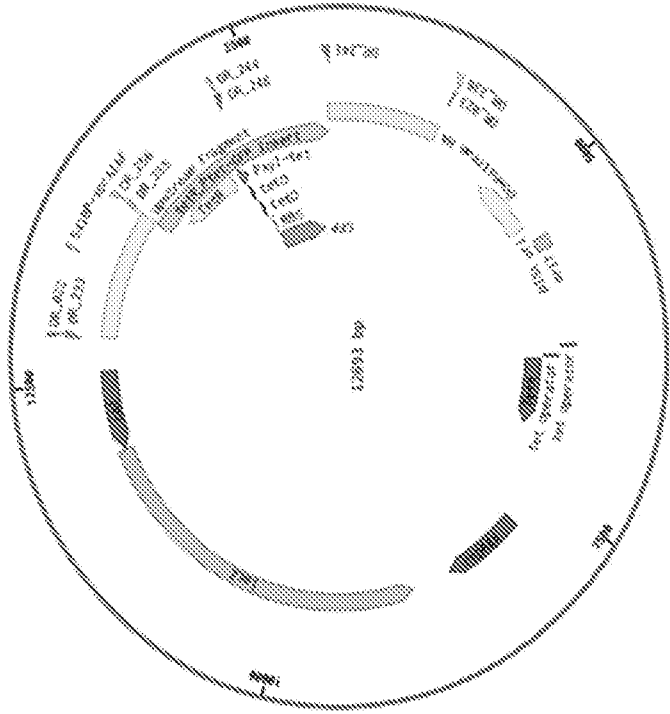
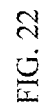
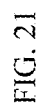


FIG. 20



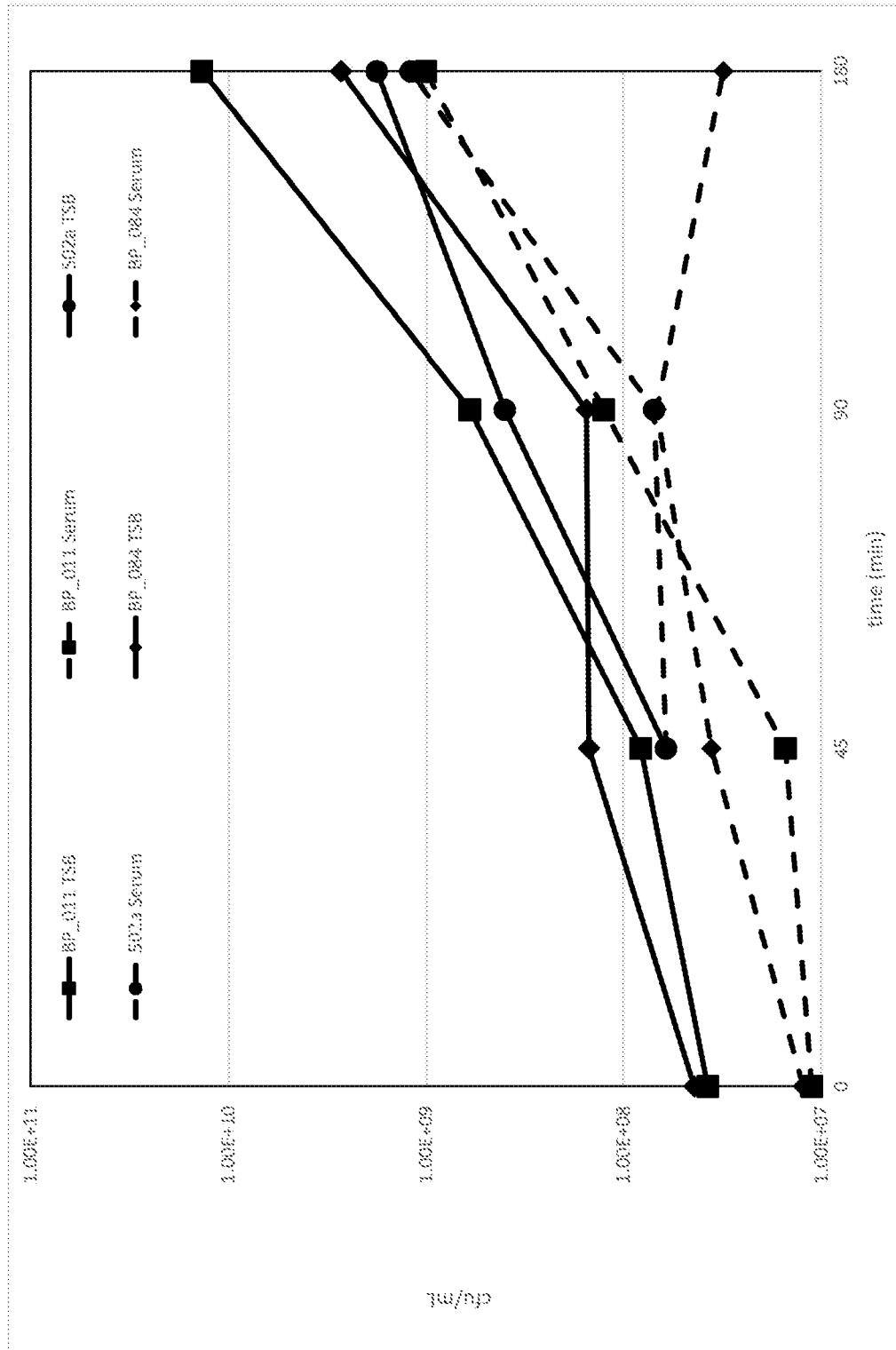


FIG. 23

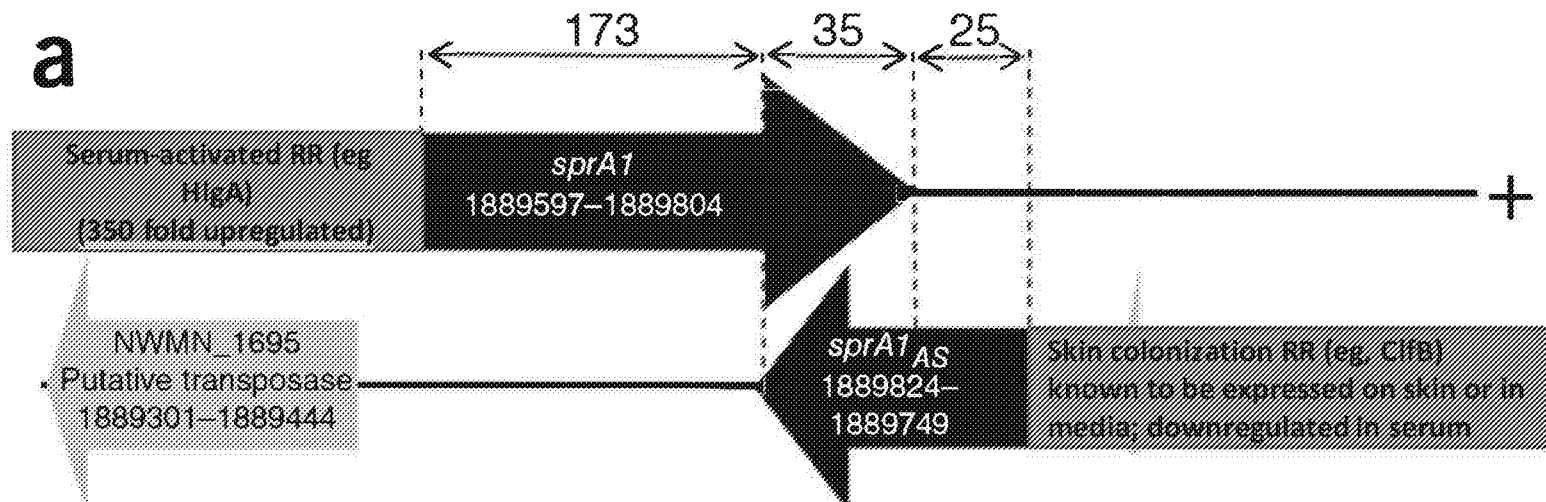


FIG. 1