(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2018 (23.08.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/152148 A1

(51) International Patent Classification:
A61K 39/085 (2006.01) *C12R 1/01* (2006.01)
A61P 31/04 (2006.01) *C12R 1/44* (2006.01)
C12N 1/21 (2006.01) *C12R 1/46* (2006.01)
C12N 15/31 (2006.01)

(21) International Application Number:
PCT/US2018/018099

(22) International Filing Date:
14 February 2018 (14.02.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/459,281 15 February 2017 (15.02.2017) US
62/529,713 07 July 2017 (07.07.2017) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

(54) Title: ENGINEERED BACTERIAL STRAIN THAT REDUCES ANTIBIOTIC-RESISTANT ENTEROCOCCUS COLONIZATION IN THE GI TRACT

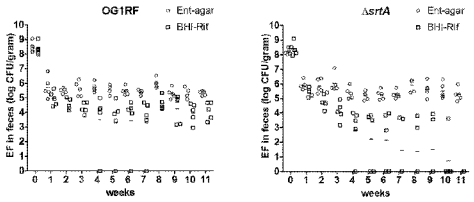

Fig. 1A

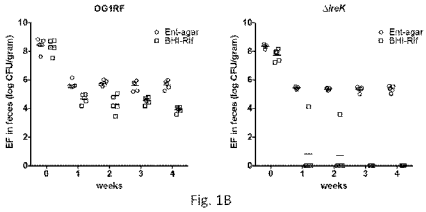

Fig. 1B

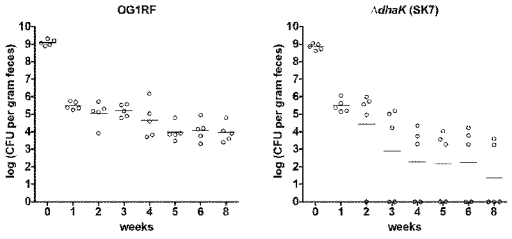

Fig. 1C

(57) Abstract: The present disclosure provides engineered modified strains of *enterococcusfaecalis* (EF) that is able to produce bacteriocin but is inefficient at establishing long-term colonization of the GI track in an animal. Compositions comprising the modified strain and methods of use are also disclosed.

[Continued on next page]

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EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

ENGINEERED BACTERIAL STRAIN THAT REDUCES ANTIBIOTIC-RESISTANT ENTEROCOCCUS COLONIZATION IN THE GI TRACT

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 62/459,281 and 62/529,713 filed on February 15, 2017 and July 7, 2017, respectively, the contents of which are incorporated by reference in their entireties.

GOVERNMENT FUNDING STATEMENT

[0002] N/A

BACKGROUND OF THE INVENTION

[0003] Bacteriocins, ribosomally synthesized antimicrobial peptides of bacterial origin, have generated interest because of their application in medical and food-based products[1-3]. Bacteriocins produced by Gram-positive lactic acid bacteria (LAB) are of particular interest due to their application as probiotics, potential treatments against infectious agents and in packaged food preservation[3,4].

[0004] The full potential of bacteriocin use in clinical settings has not been fully explored, although recent advances in delivery methods have shown some progress[5]. Antimicrobial activity of bacteriocins towards diverse bacterial species varies and can be bactericidal or bacteriostatic at different concentrations[2,6,7]. In a complex ecosystem, such as the gastrointestinal (GI) tract, bacteriocin production helps the producing strain to occupy a niche by inhibiting its competitors[8,9].

[0005] *Enterococcus faecalis* (EF), a member of the LAB, is a Gram-positive, facultative anaerobe that is a normal resident of the intestinal tract of many animals and in humans[10]. Enterococcal probiotics are used to treat irritable bowel syndrome, diarrhea, high cholesterol and to aid immune regulation[11]. Enterococcal interactions with the GI microbiota and mucosal immune system are thought to mediate their beneficial effects[12].

[0006] Despite its commensal behavior, under certain circumstances, EF can invade the host thus displaying pathogenic potential[13,14]. Enterococci are among the three

most common causes of nosocomial infections, and due to their high levels of antibiotic resistance, enterococcal infections can be difficult to treat[13,14]. Although enterococci constitute a minor fraction of the GI microbiota under normal circumstances, antibiotic treatment of the host can lead to massive overgrowth of intestinal EF and subsequent dissemination to extraintestinal sites to cause systemic EF infection [15-17].

[0007] Due to the inherent antibiotic resistance of EF, and its robust ability to acquire new drug resistance through gene transfer, new approaches are needed to prevent and treat these antibiotic-resistant nosocomial infections[16,17]. Our previous work on Bacteriocin-21 (Bac-21)-producing *Enterococcus faecalis* (EF-Bac+) in the mouse GI tract showed that the antibacterial activity of Bac-21 is primarily directed towards closely related species of enterococci, sparing the majority of the diverse population of the microbial community in the GI tract (unlike conventional antibiotics)[9,18]. This selective antimicrobial activity optimally positions EF-Bac+ as a precise tool to eliminate drug-resistant enterococci from the intestinal tract without collateral damage to the microbiota, thereby preventing the expansion, spread, and subsequent infections caused by drug-resistant enterococci.

[0008] However, one potential caveat to this approach is that the bacteriocin delivery strain itself will take up residence in the intestinal tract and may eventually acquire additional antibiotic resistance traits through a horizontal genetic exchange, re-creating the original problem[18]. A second potential caveat to this approach is that EF-Bac+ can conjugate (transmit) the bacteriocin-containing plasmid to other strains of EF in the GI tract[16].

[0009] Needed in the art is a way to overcome these challenges. In the specification below, Applicants disclose engineering an EF bacteriocin delivery strain to prevent stable colonization of the intestine and bacteriocin conjugation while retaining effective bacteriocin delivery abilities.

DESCRIPTION OF THE FIGURES

[0010] Fig 1A is a graph evaluating mice colonized with EF. Mice (N=5 per group) were given rifampicin resistant EF (OG1RF; WT or ΔsrtA) for 14 days, at which time all mice were given sterile water (Week 0). After withdrawal of EF from drinking water, fecal samples were collected and abundance of enterococci was determined by enumeration on m-Enterococcus selective agar (Ent agar), and BHI agar with rifampicin (Rif). The results shown are representative of two independent experiments. Horizontal lines indicate geometric mean. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0011] Fig. 1B is a graph evaluating mice colonized with EF. Mice (N=5 per group) were given rifampicin resistant EF (OG1RF;WT or ΔireK) for 14 days, at which time all mice were given sterile water (Week 0). After withdrawal of EF from drinking water, fecal samples were collected and abundance of enterococci was determined by enumeration on m-Enterococcus selective agar (Ent agar), and BHI agar with rifampicin (Rif). The results shown are representative of two independent experiments. Horizontal lines indicate geometric mean. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0012] Fig. 1C is a graph evaluating mice colonized with EF. Mice (N=5 per group) were given rifampicin resistant EF (OG1RF;WT or Δ*dhaK*) for 14 days, at which time all mice were given sterile water (Week 0). After withdrawal of EF from drinking water, fecal samples were collected and abundance of enterococci was determined by enumeration on m-Enterococcus selective agar (Ent agar), and BHI agar with rifampicin. The results shown are representative of two independent experiments. Horizontal lines indicate geometric mean. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0013] Fig. 2A is a bacteriocin assay by the soft agar method, with WT +pPD1 (OG1RF pPD1), Δ srtA +pPD1, ΔsrtA +pPD1::ΔbacAB bacA-E+ and ΔsrtA +pPD1::ΔbacAB. Susceptible E. faecalis (WT; OG1RF) in soft agar was overlaid on spotted test strains. Zone

of clearance around the test strains indicates the bacteriocin activity against the susceptible strain.

[0014] Fig. 2B is the summary of the conjugation assay. Four independent cultures of rifampicin resistant WT +pPD1, ΔsrtA +pPD1, ΔsrtA +pPD1::ΔbacAB, bacABCDE+ , and ΔsrtA +pPD1::ΔbacAB were mixed with spectinomycin resistant WT (OG1SP) in BHI broth at a ratio of 1:9. Samples for serial dilution were taken 24 h after the start of the experiment. Evidence of conjugation was observed by screening transconjugants via colony PCR. Numerator represents the number of transconjugants observed after screening indicated number (Denominator) of spectinomycin resistant clones. Data are representative of two biologically independent experiments. * indicates colony PCR probed for bacA gene; ** indicates colony PCR probed for bacD gene.

[0015] Fig 3 is a graph demonstrating impaired ability of one embodiment of the modified strain of the present invention to establish GI colonization. Mice (N=5) were given $\Delta srtA_r$ (rifampicin resistant) +pPD1:: ΔbacAB bacA-E+ as described in the methods and abundance was determined by enumeration on m-Enterococcus (Ent) agar and BHI agar with rifampicin (BHI-Rif). The presence of pAM401A::bacA-E + (complementing plasmid) was determined by enumerating CFU on BHI agar with rifampicin and chloramphenicol (BHI-Rif Cm). Fecal samples were obtained weekly after transition to sterile drinking water. Horizontal lines indicate geometric means. Each symbol represents an individual animal and data are representative of two biologically independent experiments. The limit of detection is 100 CFU per g feces.

[0016] Figs. 4A-4B are graphs graph showing an evaluation of mice that had been pre-colonized with vancomycin-resistant EF or or with *E. faecium*. Mice (N = 5 per group) were colonized with V583r (A) and JL277 (B; *E. faecium* 1,231,501). V583r and JL277 were removed from the drinking water of both groups two weeks before sampling (Day -3). Group 1 (A) and group 5 (B) received sterile water, whereas group 2 received $\Delta srtA_S$ (spectinomyicn resistant) (A). Group 3 (A); group 5 (B) received $\Delta srtA_S$ +pPD1:: ΔbacAB bacA-E+ in their drinking water for two additional weeks, followed by sterile water

at day 0. Fecal levels of V583r (A) and JL277 (B) were enumerated every few days. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces. The Challenger strain refers to ΔsrtA$_S$ (group2) or ΔsrtA$_S$ +pPD1:: ΔbacAB bacA-E+ (Group3 and 4).

[0017] Fig 5 diagrams the effect of the therapeutic strain on Ceftriaxone enhanced expansion of EF$_r$. Mice (N = 3 for group 2 & 3; N = 5 per group for 1) were colonized with EF$_r$. Group 1 & 2 received intraperitoneal injection of ceftriaxone for two consecutive days. Group 3 was administered saline (control). Group 2 & 3 were maintained on sterile water. Group 1 received Δ*srtA$_S$* +pPD1:: Δ*bacAB bacA-E*+ (therapeutic strain) in their drinking water 24 hours prior to ceftriaxone administration and continued to receive therapeutic strain until the end. Four days after the ceftriaxone treatment, animals were euthanized to determine the EF$_R$ levels in the tissues. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0018] Fig. 6 demonstrates that an E. faecium ΔsrtA mutant is unable to establish stable colonization of the mouse gut. Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0019] Fig. 7 shows the sequence of the partial pPD1 *ΔbacAB::ermC* vector. SEQ ID NO:1 contains part of the pPD1 backbone and includes the ermC gene insert. The ermC insert is SEQ ID NO:2. The partial pPD1 backbone sequence is SEQ ID NO:3 (taken from the full pPD1 sequence found in SEQ ID NO:10).

[0020] Fig. 8 shows the sequence of the pSK29 plasmid for expression of BacA-E in the pAM401 vector (SEQ ID NO:4). The pAM401 vector backbone is SEQ ID NO:5 and the bacA-E nucleotide sequence is SEQ ID NO:6.

[0021] Fig. 9 shows the sortase A deletion in the open reading frame of E. faecalis *srtA* gene. E. faecalis OG1RF srtA open reading frame is SEQ ID NO:7. The portion deleted in the *ΔsrtA* mutant is SEQ ID NO:8.

DESCRIPTION OF THE INVENTION

In general

[0022] Previous studies have focused on clinical isolates of enterococcus whose virulence properties are acquired by horizontal gene transfer[16,17,19]. However, these studies failed to identify specific fitness determinants essential for EF colonization of the host GI tract, which precedes infection. Because EF is a natural commensal of the GI tract, it is likely that the fundamental adaptation and survival traits required for GI colonization are encoded in the core genome of EF.

[0023] In the present invention, we disclose a member of the EF core genome that is important for stable GI colonization and exploit this knowledge to develop an improved EF-Bac+ delivery strain that is capable of eliminating drug-resistant enterococci from the mammalian GI tract but is itself impaired in bacteriocin conjugation and long-term GI colonization. These findings represent an important step towards implementation of our therapeutic strategy for the elimination of drug-resistant enterococci from the GI tract and prevention of enterococcal infections.

[0024] The Examples below show that we introduced a specific mutation into the gene cluster that encodes Bac21 bacteriocin on the pPD1 EF plasmid (full length pPD1 plasmid found in SEQ ID NO:10). When introduced into EF, the engineered pPD1 plasmid (partial sequence of pPD1 surrounding inserted gene found in SEQ ID NO:1 (inserted gene found in SEQ ID NO:2)) is not able to produce bacteriocin 21 or be transferred by conjugation to other EF by conjugation. An unexpected key finding was that when the mutant pPD1 plasmid (partial sequence of mutated plasmid, SEQ ID NO:1) is introduced into EF along with a second plasmid (SEQ ID NO:4) carrying a coding segment of *bac21* operon proximal to *bac*A promoter, in this case the *bacA-E* gene cluster (SEQ ID NO:6), the host strain of EF retains the ability to produce the bacteriocin. In the modified bacteria of the present invention, transfer of bacteriocin production to other bacteria via conjugation does not occur at detectable frequencies, which is another advantage of our system.

The present invention

[0025] In brief, the present invention typically requires a first plasmid encoding Bacteriocin 21 (Bac-21), preferably the pPD1 plasmid (SEQ ID NO:10, GenBank: KT290268, which is mutated to include SEQ ID NO:2 (partial sequence of pPD1 containing the insertion seen in SEQ ID NO:1) that is transferred to a target bacterial cell by pheromone inducible conjugation. The first plasmid will typically have a mutation in the *bacAB* sequence (e.g. insertion of *ermC* gene (SEQ ID NO:2) into pPD1 (SEQ ID NO:10 (GenBank: KT290268), to produce pPD1 ΔbacAM::ermC (partial sequence of PD1 including the mutation (insertion of SEQ ID NO:2) can be found in SEQ ID NO:1)). The target bacterial cell also comprises a second plasmid that encodes a segment proximal to the *bacA* promoter, typically *bacABCDE* (e.g., pSK29 (SEQ ID NO:4), made by insertion of *bacABCDE (bacA-E, SEQ ID NO:6)* into pAM401 vector (SEQ ID NO:5)). The target bacterial cell is an Enterococcal strain, preferably *Enterococcus faecalis* or *Enterococcus faecium,* comprising a deletion of the Sortase A gene (e.g., SEQ ID NO:9 (Δ*srtA),* made by deletion of SEQ ID NO:8 from the *E. faecalis OGIRF srtA* open reading frame (SEQ ID NO:7)).

[0026] In a preferred embodiment, the strain is EF Δ*srtA*+pPD1::Δ*bacAB bacABCDE*+. In an embodiment, the EF strain comprises ΔbacAB pPD1 (partial sequence of the mutated pPD1 plasmid found in SEQ ID NO:1), pSK29 (SEQ ID NO:4) and deletion of the *srtA* gene (SEQ ID NO:9).

[0027] In the present invention, the pPD1 plasmid has been engineered (mutated) in such a way that when present in a cell along with a second plasmid carrying a proximal segment, such as the *bacA-E* gene cluster, the host EF strain has the ability to produce the bacteriocin. However, the strain is impaired in bacteriocin conjugation and long-term GI colonization. Therefore, in one embodiment, the present invention is a bacteriocin-producing strain that is inefficient at establishing long-term colonization of the GI tract, but remains capable of delivering bacteriocin efficiently to decolonize antibiotic-resistant enterococci.

[0028] The strain will preferably have a minimized potential for transfer of bacteriocin-production traits to other bacteria via conjugation. By "minimized potential",

we refer to slight or no evidence for the transfer of *bac* operon using current methods for detection. Selection by culture techniques followed by probing for plasmid specific genes using polymerase chain reaction are the current standard methods.

Methods of creating a modified EF strain

[0029] One would first choose an EF strain to modify. Preferably, one would choose *Enterococcus faecalis* or *Enterococcus faecium*. The preferred strain used for this study does not carry mobile genetic elements and is not resistant to commonly used antibiotics. However, this invention also includes other mutants and derivatives of *E. faecalis* or other Enterococcal strains that exhibit similar or greater efficacy in delivering Bac-21 but maintain poor colonization ability.

[0030] One would then introduce a plasmid encoding the Bac21 operon. The plasmid will typically comprise a mutation in the *bacAB* sequence, as described below. This mutation may be made before or after the plasmid is introduced into the target bacterial strain. A typical Bac21-encoding plasmid is pPD1 (See Tomita, H., Fujimoto, S., Tanimoto, K. & Ike, Y. Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the Enterococcus faecalis pheromone-responsive conjugative plasmid pPD1. *Journal of bacteriology* **179**, 7843-7855 , 1997).

[0031] In one embodiment, the engineered EF bacteria of the present invention is created by first creating an in-frame deletion in *bacAB* in the target bacteria in the pPD1 backbone resulting in a pPD1 mutant plasmid (partial sequence of mutated pPD1 found in SEQ ID NO:1), which is unable to produce BAC21.

[0032] One then introduces a second plasmid construct (for example, pAM401 (SEQ ID NO:5, described below) carrying a segment proximal to the *bacA* promoter *into* the EF strain harboring the mutant pPD1. Preferably, the plasmid encodes *bacABCDE* (e.g., SEQ ID NO:4 including the bacA-E nucleotide sequence (SEQ ID NO:6). The pAM401 plasmid that carries *bacABCDE* is chloramphenicol resistant and can be referred to as the "complementing plasmid" (e.g., SEQ ID NO:4). The resulting strain that has a *bacAB* deletion in the pPD1 backbone (SEQ ID NO:10, partial sequence of mutated pPD1 can be

found in SEQ ID NO:1) and also harbors pAM401::*bacABCDE* (SEQ ID NO:4) is called the "complementing strain." This strain is now able to produce BAC21.

[0033] By "a segment proximal to the *bacA* operon," Applicants mean a segment adjacent to the *bacA* promoter that includes the coding region that is required for Bac21 production but not limited to *bacA* and *bacB* genes only. Segment *bacA-E* is proximal to *bacA* promoter compared to *bacF-I*. We found that complementing just the bacAB segment is ineffective in restoring BAC21 production. Applicants note that the first and second plasmid may be introduced into the target host in any order.

[0034] As disclosed below, Applicants successfully colonized the GI tract of mice with the complementing strain carrying the first and the second plasmids. The modified EF strain not only out-competed other strains of EF in the mouse GI tract but also eliminated an antibiotic (vancomycin) resistant strain from the GI tract, with minimal perturbation of the composition of the overall GI microbial community. Applicants tested the strains of enterococcus in the GI tract for any evidence of conjugation (transfer of the bacteriocin operon or pPD1::*ΔbacAB* plasmid) from the complementing strain to other enterococci. No conjugation was detected. Similar experiments were performed *in vitro* and no conjugation was detected.

[0035] The modified strains can be cultured industrially to provide commercial quantities of the modified strain by growth under typical bacterial fermentation conditions. The modified strains may be used in compositions in the form of intact viable cells or in a viable lyophilized form.

Method of Use

[0036] We envision that this product would be used in a prophylactic manner, to eliminate GI colonization by drug-resistant (e.g., antibiotic-resistant) enterococci in patients at risk of acquiring healthcare-associated enterococcal infections. In such a scenario, live organisms of the therapeutic strain would be delivered to patients orally, potentially via suspension in a beverage or food product or in the form of lyophilized

organisms formulated in a pill. One could follow prior art methods to determine a correct dosage. For example, one may consult US Pat. No. 5,728,380; EP0508701, or US20070098744, the contents of which are incorporated by reference.

[0037] Other routes of administration are also contemplated that allow for the product to reach the gut lumen, e.g., but not limited to, rectally, G-tube, NG-tube, direct administration to the intestinal track (gut lumen), etc.

[0038] In one embodiment, the patient is preferably a human patient.

[0039] In addition, veterinary use of the present modified strains, products and compositions are contemplated. By veterinary use, we mean use in a non-human animal, including domesticated animals, livestock and wild animals in captivity.

[0040] Non-human animals include, but are not limited to domesticated animals, livestock and also wild animals kept in captivity (e.g., housed in zoos or sanctuaries). Domesticated animals includes, without limitation, cats, dogs, rabbits, guinea pigs, ferrets, hamsters, mice, gerbils, horses, cows, goats, sheep, donkeys, pigs, and the like. Livestock includes animals raised for production of food products, including, but not limited to, poultry, pigs, cows, buffalo, sheep, goat, and the like. Wild animals contemplated include animals that are found in the wild or housed at zoos, sanctuaries or wild-life habitats.

[0041] In a preferred embodiment, the non-human animals are birds, for example, domesticated birds, pet birds and poultry. Suitable domesticated or pet birds include, but are not limited to, for example, parakeets, cockatiels, lovebirds, pigeons, parrotlets, caiques, small conures, lories, lorikeets, canaries, parrots, crows, doves, toucans, macaws, cockatoos, and the like.

[0042] In a more preferred embodiment, the birds are poultry. Suitable poultry able to be treated by the modified strains and methods described herein include, but are not limited to, chickens, turkeys, pheasants, ducks, geese, partridge, quail and the like.

[0043] It is specifically contemplated that the methods described herein are able to reduce or eliminate enterococcal colonization (specifically antibiotic-resistant enterococcal colonization) of the GI track of poultry to be slaughtered and consumed. The ability to reduce or eliminate enterococcal colonization reduces the likelihood of the poultry to result in disease in humans.

[0044] In addition, non-human animals, including livestock, particularly chickens, are commonly colonized by enterococci, including enterococcal species that cause disease in humans (i.e., *E. faecalis* and *E. faecium*) as well as enterococcal species that cause disease in the animals themselves (i.e., *E. cecorum*). This therapeutic strategy may be used to reduce or eliminate enterococcal colonization of animals to reduce the enterococcal burden in the animal GI tract and minimize the potential for: (i) transmission of antibiotic-resistant, animal-derived enterococci (or antibiotic-resistance genes harbored by these enterococci) to humans via contamination of uncooked food products; and (ii) disease of the animal host resulting from spread of pathogenic enterococci.

[0045] A method of treating enterococci infections in a human patient in need thereof is contemplated in some embodiments. The method comprises administering to the human patient a therapeutically effective amount of the modified enterococcal strain or a composition comprising the modified strain.

[0046] Methods of treating enterococci infections in a non-human animal are also contemplated herein. The methods comprise administering to the non-human animal a therapeutically effective amount of the modified enterococcal strain or compositions described herein. In a preferred embodiment, the non-human animal is a poultry, more specifically a chicken, turkey, pheasant, quail, duck or the like. In a preferred embodiment, the non-human animal is a chicken.

[0047] The term "treat", "treating", and "treatment" of bacterial infections, including enterococcal infections, encompasses, but is not limited to, reducing, inhibiting, alleviating, improving, delaying or limiting at least one symptom of the infection, for example, may be characterized by one or more of the following: (a) reducing, slowing or

inhibiting growth of drug-resistant bacteria (e.g., antibiotic-resistant enterococcal infections) within the GI track (intestine) of the animal, (b)reducing or preventing bacterial colonization of the GI track of the animal by a pathogenic or drug-resistant strain of bacteria (e.g., antibiotic-resistant enterococci), (c) reducing or eliminating enterococcal colonization of the GI track of the animal, (d) reducing the enterococcal burden in the GI track of the animal, (e) reducing or inhibiting the transmission of antibiotic-resistant, animal derived enterococci from livestock to humans, and(f) reducing or eliminating one or more symptoms of infection or GI colonization associated with drug-resistant bacteria in the animal.

[0048] The term "effective amount" or “therapeutically effective amount” as used herein refer to an amount sufficient to effect beneficial or desirable biological, therapeutic and/or clinical results. In one embodiment, the "effective amount" is an amount sufficient to reduce, inhibit, or eliminate the colonization of enterococci bacteria, e.g., antibiotic-resistant enterococci, within the GI track of an animal. An effective amount refers to the quantity of the modified strain (complementary strain) sufficient to yield a desired therapeutic response without undue adverse side effects such as toxicity, irritation, or allergic response. The specific “effective amount” will, obviously, vary with such factors as the particular condition being treated, the physical condition of the subject, the type of animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

[0049] Those skilled in the treatment of bacterial infections, in particular enterococcal infections, will easily determine the therapeutically effective amount of the modified stain or complementary strain to be used. It may be appropriate to administer the therapeutically effective dose in the form of two or more sub-doses at appropriate intervals throughout the day.

[0050] The exact dosage and frequency of administration depends on the particular composition, the particular condition being treated, the severity of the condition

being treated, the age, weight and general physical condition of the particular animal as well as the other medication, the animal may be taking, as is well known to those skilled in the art.

[0051] Suitable embodiments of the present invention provide for daily, weekly or monthly doses of the modified strain or composition described herein. In some embodiments, the strain or compositions are administered in effective daily dosages. Furthermore, in some embodiments, the effective daily amount may be lowered or increased depending on the response of the treated animal and/or depending on the evaluation of the physician or veterinarian prescribing the compounds of the instant invention. The effective daily amount ranges mentioned hereinabove are therefore only guidelines.

[0052] In some embodiments, methods of inhibiting the GI colonization by antibiotic-resistant enterococci in human patient in need thereof are contemplated. The method comprises administering to the patient a therapeutically effective amount of the modified enterococcal strain or compositions comprising the modified enterococcal strain.

[0053] In some embodiments, the modified strain and compositions are used to prophylactically to inhibit the colonization of the GI track of an animal (human or non-human) by pathogenic or drug-resistant (antibiotic-resistant) bacteria (e.g., antibiotic-resistant enterococci). The term "prophylactically" refers to the ability to inhibit the colonization of the GI track with pathogenic or drug-resistant bacteria or to prevent or inhibit the overgrowth of pathogenic or drug-resistant bacteria within the GI track of the animal.

[0054] Another embodiment contemplates methods of altering the microbial composition of the GI track of a human or non-human animal or patient, the method comprising administering to the human or non-human animal or patient a therapeutically effective amount of the modified enterococcal strain or the compositions described herein in order to alter the microbial composition of the GI track. "Altering the microbial composition of the GI track" refers to the ability to alter the composition and make-up of

the microbes inhabiting the GI track. In a preferred embodiment, the GI track is altered to have one or more of the following characteristics: (a) increase in the amount of beneficial bacteria within the GI track, (b) increase in the ratio of beneficial bacteria to drug-resistant bacteria (e.g., greater that 2:1 ratio), (c) reduce or decrease the amount of drug-resistant (e.g., antibiotic-resistant) bacteria within the GI track. In some preferred embodiments, the non-human animal is a poultry, for example, but not limited to, a chicken.

[0055] Further embodiments describe methods of preventing GI track colonization of antibiotic resistant enterococci in a human patient comprising administering to the patient a therapeutically effective amount of the modified enterococcal strain or composition described herein to prevent GI track colonization.

[0056] Some embodiments describe a method of treating enterococci infections in a non-human animal comprising administering to the non-human animal a therapeutically effective amount of the modified enterococcal strain or compositions described herein in order to treat the enterococci infection. In some embodiments, the preferred non-human animal is a poultry, in some embodiments, a chicken.

[0057] In some embodiments, methods of preventing GI track colonization of antibiotic resistant enterococci in a non-human animal are contemplated. Preventing GI track colonization encompasses the ability to prevent the overgrowth of antibiotic resistant enterococci in the GI track, as demonstrated by a reduced number or a reduced ratio of antibiotic resistant enterococci to non-harmful bacteria within the GI track. The method comprises administering an effective amount of the modified strain or compositions described herein in order to prevent GI track colonization.

[0058] The methods of administration described herein may be done in a single dose or over multiple dosages over a number of days, weeks or months depending on the treatment necessary. In some embodiments, the methods are performed daily. Daily administration includes single administration or multiple administrations within the day to provide the effective amount. In other embodiments, administration is performed weekly or monthly.

[0059] In another embodiment, the disclosure provides a method of reducing contamination of livestock food products with antibiotic-resistant enterococci. The method comprises administering to the livestock before slaughter an effective amount of the modified enterococcal strain or the composition described herein in an amount effective to reduce the amount of antibiotic-resistant enterococci in the GI track of the livestock. The reduction of antibiotic-resistant enterococci in the GI track of livestock, in turn reduces the contamination of livestock food products when the livestock is slaughtered and prepared for consumption. In some embodiments, the complementary strain (modified strain) or composition described herein is administered at least once prior to slaughter of the livestock. In other embodiments, the complementary strain (modified strain) or composition described herein may be administered over multiple days or weeks prior to slaughter. In one embodiment, the livestock are administered the complementary strain (modified strain) or composition described herein for at least two days prior to slaughter, alternatively for at least three days prior to slaughter, alternatively for at least four days prior to slaughter, alternatively for at least five days prior to slaughter, alternatively for at least six days prior to slaughter, alternatively for at least seven days prior to slaughter, alternatively for at least 2 weeks prior to slaughter, alternatively for at least 3 weeks prior to slaughter, alternatively for at least 4 weeks prior to slaughter. One skilled in the art will be able to determine the correct administration in order to reduce the contamination of livestock food product by reducing or eliminating the enterococci colonization in the GI track of the livestock animal.

[0060] In a preferred embodiment, the disclosure provides a method of reducing contamination of poultry food products with enterococci, more specifically antibiotic-resistant enterococci. The method comprises administering to the poultry before slaughter an effective amount of the modified enterococcal strain or the composition described herein in an amount effective to reduce or eliminate the amount of antibiotic-resistant enterococci in the GI track of the poultry. The reduction of antibiotic-resistant enterococci in the GI track of the poultry, in turn reduces the contamination of poultry food products when the poultry is slaughtered and prepared for consumption. In a preferred embodiment, the poultry is a chicken.

Compositions

[0061] The modified strains disclosed herein can be formulated into compositions, include feed compositions. Feed compositions include feed compositions for human consumption or non-human animal consumption. Food compositions for human consumption are any compositions formulated for ingestion by a human being. Suitable non-human compositions include, for example livestock feed, pet food, pet food compositions, animal food, animal food compositions, which are intended for ingestion by a non-human animal.

[0062] Another embodiment of the present invention is feed compositions and methods of producing feed composition for non-human animals (e.g., livestock or domesticated animals). The feed composition comprising the modified enterococcal strain (e.g., complementing strain carrying the first and second plasmids) may be formulated using at least one animal feed product. The feed composition is made by mixing a sufficient amount of the modified enterococcal stain with feed product or water. In some embodiments, the resulting mixture is granulated and/or dried to obtain a powder, pellets, granules, gels or other solid forms of feed. In other embodiments, the feed composition is provided in a gel or water composition. In some embodiments, the modified strain is lyophilized before being added to the feed product to provide solid forms of the feed composition. This feed composition may be a solid formulation or liquid formulation that may further comprise one or more compatible carriers or diluents. In a preferred embodiment, the feed composition is suitable for poultry (e.g., poultry feed). In some embodiments, the feed is chicken feed.

[0063] In some embodiments, the modified strain is formulated into compositions comprising a pharmaceutically acceptable carrier. Suitable carriers are carriers that do not affect the viability of the modified strain within the composition. Suitable formulations of the composition may be used for veterinary use and administration, or for human use and administration.

[0064] In some embodiments, the modified strains are formulated into compositions as dietary supplements for human or non-human animal consumption or in treatment formulations. In some embodiments, the dietary supplements are contemplated to be ingested in addition to the normal diet of the animal.

[0065] Suitable examples of compositions for use as a dietary supplement, include, but are not limited to, for example, a gravy, drinking water, beverage, liquid concentrate, yogurt, powder, granule, paste, suspension, chew, morsel, treat, snack, pellet, pill, capsule, tablet, or any other delivery form. The dietary supplements can be specially formulated for consumption by a particular animal. In one detailed embodiment, the dietary supplement can comprise a high concentration of complementary strain such that the supplement can be administered to the animal in small amounts, or in the alternative, can be diluted before administration to an animal. The dietary supplement may require admixing with water or a suitable diluent prior to administration to the animal.

[0066] The composition may be refrigerated or frozen. The modified strains may be pre-blended with the other components of the composition to provide the beneficial or effective amounts needed, may be coated onto a food composition, dietary supplement, or food product formulated for human or non-human consumption, or may be added to the composition prior to offering it to the human or non-human animal, for example, using a powder or a mix.

[0067] Food compositions may be formulated in one embodiment in to contain the modified strains in the range of about 10^2 to about 10^{11} colony forming units (CFU) per gram of the composition. Dietary supplements may be formulated to contain several fold higher concentrations of the modified strain, to be amenable for administration to an animal in the form of a tablet, capsule, liquid concentrate, or other similar dosage form, or to be diluted before administration, such as by dilution in water or diluent, spraying or sprinkling onto a food, and other similar modes of administration.

[0068] In another embodiment, the composition comprising the modified enterococcal stain may be formulated for administration with the animal feedstuff as a concentrated feed additive or a premix may be prepared with the normal animal feed.

[0069] Aspects of the present disclosure that are described with respect to methods can be utilized in the context of the compositions or kits discussed in this disclosure. Similarly, aspects of the present disclosure that are described with respect to the compositions can be utilized in the context of the methods and kits, and aspects of the present disclosure that are described with respect to kits can be utilized in the context of the methods and compositions.

[0070] This disclosure provides kits. The kits can be suitable for use in the methods described herein. In one embodiment, the kit comprises the modified strain and instructions for use. The instructions may provide recommended modes of administration to the human or non-human animal, including, but not limited to, specifying dosage, quantity and frequency of administration.

[0071] In some embodiments, the present disclosure provides a kit for mixing the modified stain of enterococci with non-human animal feed to make animal feed composition for animal consumption. The kit may include instructions on the ration of modified strain to animal food product and methods of mixing suitable for administration.

[0072] It should be apparent to those skilled in the art that many additional modifications beside those already described are possible without departing from the inventive concepts. In interpreting this disclosure, all terms should be interpreted in the broadest possible manner consistent with the context. Variations of the term "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, so the referenced elements, components, or steps may be combined with other elements, components, or steps that are not expressly referenced. Embodiments referenced as "comprising" certain elements are also contemplated as "consisting essentially of" and "consisting of" those elements. The term "consisting essentially of" and "consisting of" should be interpreted in line with the MPEP and relevant Federal Circuit's

interpretation. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. "Consisting of" is a closed term that excludes any element, step or ingredient not specified in the claim.

[0073] The following non-limiting examples are included for purposes of illustration only, and are not intended to limit the scope of the range of techniques and protocols in which the compositions and methods of the present invention may find utility, as will be appreciated by one of skill in the art and can be readily implemented. The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

EXAMPLES

Materials and Methods

[0074] **Bacterial strains growth media and reagents**. The strains used in this study are listed in Table 1. Brain-heart infusion medium (BHI) and m-Enterococcus agar (Ent-agar) (Difco) were prepared as described by the manufacturer (Becton Dickinson). Antibiotics were purchased from Sigma and used at the following concentrations: rifampicin, 200 g ml–1; spectinomycin, 500 g ml–1; chloramphenicol, 15 g ml–1. *E. faecalis* was cultured in BHI media at 37 ºC. All restriction enzymes were purchased from New England BioLabs. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for all PCRs performed for strain and plasmid construction. Oligonucleotides were synthesized by Fisher Scientific.

Table 1

Strains	Description	Reference or source
OG1sp	Spontaneous mutant of OG1; Sp^r	9
OG1RF	Spontaneous mutant of OG1; Rif^r, Fa^r	9

JL277	1,231,501 *rpoB* H486Y; Rif^r derivative	9
IB1 (V583$_r$)	V583 spontaneous Rif^r derivative, Van^r	9
IB10(Δ*srtA*$_r$)	OG1RF Δ*srtA*	unpublished
IB16 (Δ*srtA*$_s$)	OG1SP Δ*srtA*	unpublished
CK119	OG1RF Δ*ireK*	26
SK7	OG1RF Δ*dhAK* (inframe Deletion in OG1RF_11146 to OG1RF_11149)	unpublished
IB10 pPD1	OG1RF Δ*srtA*pPD1	This study
SK16 pSK33	OG1RF Δ*srtA* pPD1:: Δ*bacAB*	This study
SK17 pSK33	OG1SP Δ*srtA* pPD1:: Δ*bacAB*	This study
SK16 pSK33 pSK29	OG1RF Δ*srtA* pPD1:: Δ*bacAB* pAM401::bacABCDE+	This study
SK17 pSK33 pSK29	OG1SP Δ*srtA* pPD1:: Δ*bacAB* pAM401::*bacABCDE*+	This study
Plasmids		
pPD1	Bac-21, pheromone-inducible conjugative plasmid	9
pSK33	*ΔbacAB::ermC* in pPD1	9
pSK29	*bacABCDE* in pAM401	9
pCJK218	*E. faecalis* allelic exchange vector (Cmr); pheS* counterselection	20
pCJK245	*E. faecalis* allelic exchange vector (Cmr); thyA* counterselection	21

[0075] **Animals.** The committee for animal care and use at the Medical College of Wisconsin approved all animal-related procedures and experiments. Five-week- old male C57Bl/6 mice were obtained from Jackson Laboratories (West-RB08 barrier). Upon arrival, mice were allowed to adapt to the new environment for at least one week before the start of any experiment. Animals were housed under specific-pathogen-free conditions in the Medical College of Wisconsin vivarium. Experimental sample sizes were determined by appropriate husbandry considerations as determined by the Medical College of Wisconsin vivarium and experiments were repeated as described. No blinding was performed, and no scheme of randomization was applied when allocating mice for the experiments.

[0076] **Generation of Δ*srtA* and Δ*dhaK* mutants.** In-frame deletion mutants in *E. faecalis* were constructed using markerless allelic exchange as previously described[20,21].

Mutant alleles were constructed and introduced into pCJK218 or pCJK245 using Gibson assembly[22]. To ensure unperturbed expression of neighboring genes, 5' and 3' end codons of the gene were retained in each deletion allele.

[0077] **Generation of Δ*srtA* +pPD1 and Δ*srtA*+pPD1::Δ*bacAB bacABCDE*+.** pPD1 was introduced into the EF Δ*srtA* mutant (IB10; Δ$srtA_R$ and IB16; Δ$srtA_S$) via conjugation using a donor strain; WT+pPD1 (OG1SP+pPD1 or OG1RF+pPD1 respectively). The resulting transconjugants are Δ*srtA* +pPD1. To construct Δ*srtA*+pPD1::Δ*bacAB bacABCDE*+, markerless allelic exchange was used to introduce in-frame deletion of *srtA* in OG1RF+pPD1::Δ*bacAB bacABCDE*+ or OG1SP+pPD1::Δ*bacAB bacABCDE*+ strains, as previously described[21]. PCR analysis and bacteriocin assay confirmed the resulting strains.

[0078] **Bacteriocin Assays.** As previously described[23], bacteriocin-producing or bacteriocin-sensitive indicator strains are spotted on BHI agar and incubated at 37 ºC for 30 minutes to dry the spots. 50 μl of an overnight culture of the indicator strain (OG1RF or OG1sp) grown in BHI broth was added to 5 ml of molten BHI soft agar to overlay evenly onto the BHI agar containing spots of test strains. Zones of inhibition of the susceptible strain around the test strain spots were monitored after overnight incubation at 37 ºC.

[0079] **Mouse colonization studies**. As previously described[9], overnight cultures of *E. faecalis* with appropriate antibiotics were washed with sterile water and added to autoclaved water to a final concentration of 5 X 10^8 CFU ml–1. Persistence of *E. faecalis* in drinking water was determined daily and remained between 10^7 and 10^8 CFU ml–1 over three days. Drinking water was changed every 3–4 days to maintain the appropriate inoculum and mice were allowed to drink ad libitum. After two weeks, the inoculated drinking water was replaced with sterile water for the duration of the experiment. For the sequential colonization experiment, mice were first colonized with an initial strain by feeding the bacteria through drinking water for two weeks. Three days after the initial strain was withdrawn from drinking water, the challenger strain was introduced in the drinking water for two weeks, after which animals were returned to regular sterile water (day 0).

[0080] **Bacterial culture and quantification of EF from mouse feces and intestines.** Indigenous enterococcal levels in all experimental animals were determined by culturing feces on m-Enterococcus agar (Ent-agar) to determine baseline colonization levels prior to introducing the experimental strain. Abundance of the experimental strain in feces and homogenized tissue samples were enumerated as previously described on BHI agar plates with appropriate antibiotics[9]. To enumerate indigenous enterococci or total enterococci, fecal and tissue homogenates were plated in serial dilution on m-Enterococcus agar (Difco).

[0081] **In-vitro Conjugation Assay.** Donor (rifampicin resistant strains) and recipient (spectinomycin resistant WT; OG1SP) strains were grown separately in BHI media at 37°C for 16 hours. The cell cultures were diluted 1:5 in fresh media to use for subsequent inoculation. Donor and recipient cell cultures were mixed at 1:9 ratios. Samples for serial dilution were taken 24 h after the start of the experiment. Evidence of conjugation was observed by screening transconjugants via colony PCR. To determine transconjugants, colonies were selected and used as PCR templates for identification of the *bacA* or *bacD* gene using gene-specific primers as previously described[9]. PCR products were identified using agarose gel electrophoresis.

Results and Discussion

[0082] Our previous work provided proof-of-concept that EF can deliver bacteriocin to specifically eliminate multidrug-resistant enterococci from the GI tract[9,18]. However, one potential caveat to this approach is that the bacteriocin delivery strain itself will take up residence in the intestinal tract and may eventually acquire additional antibiotic resistance traits through horizontal genetic exchange, re-creating the original problem. To overcome this obstacle, we sought to engineer a bacteriocin-producing strain that is inefficient at establishing long-term colonization of the GI tract, but remains capable of delivering bacteriocin efficiently to decolonize antibiotic-resistant enterococci. An additional consideration was to minimize the potential for transfer of bacteriocin-production traits to other bacteria via conjugation.

[0083] We began by examining several factors encoded in the core EF genome that we hypothesized, based on our unpublished studies and previously published work, to be important for colonization and persistence of EF in the GI tract. These included Sortase A (SrtA, OG1RF_12327), IreK (OG1RF_12384), and the DhaK pathway (OG1RF_11146-11149). SrtA is a key membrane-anchored transpeptidase that mediates attachment of proteins to the cell-surface. Several studies on sortase[24,25] and associated cell-wall proteins (unpublished data) provided evidence for their roles in bacterial physiology in response to the extracellular environment. Moreover, SrtA is important for the establishment of enterococcal urinary tract infections[25]. Collectively, these observations suggest that SrtA might also play a role in interactions with components of the intestinal milieu during GI colonization. IreK is a transmembrane kinase required for intrinsic enterococcal resistance to antimicrobial stresses that affect the cell envelope, including stresses likely to be encountered in the GI tract such as lysozyme and cholate[26]. A preliminary study indicated that IreK contributed to short-term persistence in the mouse GI tract[26], and recently a role for IreK in stable GI colonization has been described (Banla et al, (2017) Infect. Immun. 86(1). pii: e00381-17. doi: 10.1128/IAI.00381-17). The DhaK pathway mediates glycerol utilization by EF and is upregulated in the intestinal environment[27] (unpublished data), suggesting that adaptation of metabolism is required for GI colonization. Individual mutants of EF (Δ*srtA*, Δ*ireK*, or Δ*dhaK*,) lacking these factors exhibited a reduced ability to colonize the GI tract compared to the wild-type (WT) (Fig. 1), documenting a role for each as an enterococcal GI colonization factor.

[0084] An EF strain defective in GI colonization must retain the ability to produce Bac21 to be therapeutically effective. To assess this capacity, we focused on the Δ*srtA* mutant. First, we constructed a Δ*srtA* mutant carrying two plasmids that, when present together, enable Bac-21 production by the host EF strain[9]. EF Δ*srtA*+pPD1::Δ*bacAB bacABCDE*+ (therapeutic strain) was effective at inhibiting growth of susceptible EF both in liquid culture (data not shown) and on solid media in vitro (Fig. 2A), similar to WT+pPD1 (OG1RF +pPD1) and Δ*srtA*+pPD1, confirming that the Δ*srtA* mutation does not impact the ability of the EF strain to produce Bac-21. The benefit of using two separate plasmids to encode the bacteriocin is threefold: first, the Δ*bacAB* mutant of pPD1 exhibits reduced

ability to transfer by conjugation[9]; second, because the bacteriocin production operon is separated into 2 independent loci the chances of transferring Bac-21 production capability to other EF strains is reduced (as it would require simultaneous transfer of both loci); and third, growth in the intestine leads to spontaneous loss of one of the plasmids (Fig 3), leaving an EF strain carrying only pPD1::Δ*bacAB*, which we have previously shown to exhibit a defect in intestinal persistence[9].

[0085] To confirm that conjugation was impaired in the Δ*srtA* mutant, we performed conjugation assays in vitro. The therapeutic strain was severely impaired in its ability to transfer pPD1::Δ*bacAB* to the recipient strain (Fig. 2B), as desired. Two factors contribute to the impaired conjugation ability in this strain: first, although the mechanism is unclear, we previously found that pPD1::Δ*bacAB* is inherently unable to transfer by conjugation efficiently[9]; second, SrtA was previously shown to be important for cell-cell aggregation and conjugation[28].

[0086] The improved "therapeutic" strain we developed (Δ*srtA*+pPD1::Δ*bacAB bacABCDE*+) therefore exhibits multiple desirable properties: production of Bac21 (Fig 2), impaired ability to conjugate (Fig 2), and impaired ability to establish stable GI colonization (Figs 1, 3). To examine the ability of this strain to eliminate drug-resistant enterococci from the GI tract, we delivered it to mice that had been pre-colonized with vancomycin-resistant *E. faecalis* (V583) or a clinical isolate of *E. faecium* (JL277; 1,231,501) (Fig. 4). Compared to the control group, V583 and *E. faecium* levels in the challenged mice were below the detection limit in most of the animals after treatment with the improved therapeutic strain (Fig. 4). In addition to clearing the MDR-strains of enterococci, this therapeutic strain would eventually be lost due to its inability to persist longer (anticipated result; Fig. 4).

[0087] In this study, we expanded on our initial findings to develop an *E. faecalis* strain with the potential to be a useful therapeutic in humans and animals with the following traits: able to secrete Bac-21, unable to transfer bacteriocin-producing trait to other bacteria, and unable to maintain long-term colonization in the GI tract itself.

[0088] This invention can be extended to other mutants and derivatives of *E. faecalis* that exhibit similar or greater efficacy in delivering Bac-21 but maintain poor colonization ability. We envision that this product would be used in a prophylactic manner, to eliminate GI colonization by antibiotic-resistant enterococci in patients at risk of acquiring healthcare associated enterococcal infections. In such a scenario, live organisms of the therapeutic strain would be delivered to patients orally, potentially via suspension in a beverage or food product, or in the form of lyophilized organisms formulated in a pill. In addition, livestock, particularly chickens, are commonly colonized by enterococci. This therapeutic strategy also has the potential to be used to eliminate enterococcal colonization of food animals prior to harvest and processing to reduce the enterococcal burden in the animal GI tract and minimize the potential for transmission of antibiotic-resistant, animal-derived enterococci (or antibiotic-resistance genes harbored by these enterococci) to humans via contamination of uncooked food products.

[0089] Enterococci are intrinsically resistant to broad-spectrum cephalosporin antibiotics. They expand in the gut and disseminate systemically causing infections in immunocompromised individuals and hospitalized patients undergoing cephalosporin therapy. We examined the effect of this improved therapeutic strain on the antibiotic expansion of EF_R (V583 and OG1RF). To the mice that were pre-colonized with EF_R, the therapeutic strain was delivered in drinking water 24 hours prior to the administration of ceftriaxone and continued until the end. As demonstrated in Fig. 5, the expansion of EF_R was compared to the control groups that were not treated with therapeutic strain but, received ceftriaxone or saline respectively (group 2 and 3). EF_R expansion was 4-fold lower in therapeutic treated – ceftriaxone-treated mice (group 1), compared to the group 2. In addition, dissemination of EF_R and the therapeutic strain to the spleen and liver was not observed. This observation suggests that our modified therapeutic strain has the potential to minimize the antibiotic (ceftriaxone)- induced expansion of EF_R and prevent the chance of translocation of enterococci across the GI barrier.

[0090] As demonstrated in Fig. 6, an E. faecium ΔsrtA mutant is unable to establish stable colonization of the mouse gut. Gut colonization was monitored in mice

(N=5 per group). Fecal samples were collected and abundance of enterococci was determined by enumeration on BHI agar with rifampicin (the E. faecium strains were marked with rifampicin resistance). Each symbol represents an individual animal. The limit of detection is 100 CFU per g feces.

[0091] References

1. Perez RH, Zendo T, Sonomoto K: **Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications**. *Microbial cell factories* 2014, **13 Suppl 1**:S3.
2. Cotter PD, Ross RP, Hill C: **Bacteriocins - a viable alternative to antibiotics?** *Nat Rev Microbiol* 2013, **11**(2):95-105.
3. Ahmad V, Khan MS, Jamal QM, Alzohairy MA, Al Karaawi MA, Siddiqui MU: **Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation**. *Int J Antimicrob Agents* 2016.
4. Dobson A, Cotter PD, Ross RP, Hill C: **Bacteriocin production: a probiotic trait?** *Applied and environmental microbiology* 2012, **78**(1):1-6.
5. Arthur TD, Cavera VL, Chikindas ML: **On bacteriocin delivery systems and potential applications**. *Future Microbiol* 2014, **9**(2):235-248.
6. Jack RW, Tagg JR, Ray B: **Bacteriocins of gram-positive bacteria**. *Microbiol Rev* 1995, **59**(2):171-200.
7. Hammami R, Fernandez B, Lacroix C, Fliss I: **Anti-infective properties of bacteriocins: an update**. *Cellular and molecular life sciences: CMLS* 2013, **70**(16):2947-2967.
8. Zheng J, Ganzle MG, Lin XB, Ruan L, Sun M: **Diversity and dynamics of bacteriocins from human microbiome**. *Environmental microbiology* 2015, **17**(6):2133-2143.
9. Kommineni S, Bretl DJ, Lam V, Chakraborty R, Hayward M, Simpson P, Cao Y, Bousounis P, Kristich CJ, Salzman NH: **Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract**. *Nature* 2015, **526**(7575):719-722.

10. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Jr., Gilmore MS: **Genetic diversity among Enterococcus faecalis**. *PloS one* 2007, **2**(7):e582.
11. Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ, von der Weid T: **Supplementation of food with Enterococcus faecium (SF68) stimulates immune functions in young dogs**. *J Nutr* 2003, **133**(4):1158-1162.
12. Pieniz S, Andreazza R, Anghinoni T, Camargo F, Brandelli A: **Probiotic potential, antimicrobial and antioxidant activities of Enterococcus durans strain LAB18s**. *Food Control* 2014, **37**:251-256.
13. Richards MJ, Edwards JR, Culver DH, Gaynes RP: **Nosocomial infections in combined medical-surgical intensive care units in the United States**. *Infect Control Hosp Epidemiol* 2000, **21**(8):510-515.
14. Fisher K, Phillips C: **The ecology, epidemiology and virulence of Enterococcus**. *Microbiology* 2009, **155**(Pt 6):1749-1757.
15. Shepard BD, Gilmore MS: **Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance**. *Microbes and Infection / Institut Pasteur* 2002, **4**(2):215-224.
16. Broaders E, Gahan CG, Marchesi JR: **Mobile genetic elements of the human gastrointestinal tract: potential for spread of antibiotic resistance genes**. *Gut microbes* 2013, **4**(4):271-280.
17. Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A: **Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2010, **16**(6):541-554.
18. Kommineni S, Kristich CJ, Salzman NH: **Harnessing bacteriocin biology as targeted therapy in the GI tract**. *Gut microbes* 2016:0.
19. Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF *et al*: **Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis**. *Science* 2003, **299**(5615):2071-2074.

20. Vesic D, Kristich CJ: **A Rex family transcriptional repressor influences H2O2 accumulation by Enterococcus faecalis**. *Journal of bacteriology* 2013, **195**(8):1815-1824.

21. Kristich CJ, Djoric D, Little JL: **Genetic basis for vancomycin-enhanced cephalosporin susceptibility in vancomycin-resistant enterococci revealed using counterselection with dominant-negative thymidylate synthase**. *Antimicrobial agents and chemotherapy* 2014, **58**(3):1556-1564.

22. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases**. *Nat Methods* 2009, **6**(5):343-U341.

23. Maricic N, Dawid S: **Using the overlay assay to qualitatively measure bacterial production of and sensitivity to pneumococcal bacteriocins**. *J Vis Exp* 2014(91):e51876.

24. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ: **Contribution of autolysin and Sortase a during Enterococcus faecalis DNA-dependent biofilm development**. *Infection and immunity* 2009, **77**(9):3626-3638.

25. Kemp KD, Singh KV, Nallapareddy SR, Murray BE: **Relative contributions of Enterococcus faecalis OG1RF sortase-encoding genes, srtA and bps (srtC), to biofilm formation and a murine model of urinary tract infection**. *Infection and immunity* 2007, **75**(11):5399-5404.

26. Kristich CJ, Wells CL, Dunny GM: **A eukaryotic-type Ser/Thr kinase in Enterococcus faecalis mediates antimicrobial resistance and intestinal persistence**. *Proc Natl Acad Sci U S A* 2007, **104**(9):3508-3513.

27. Lindenstrauss AG, Ehrmann MA, Behr J, Landstorfer R, Haller D, Sartor RB, Vogel RF: **Transcriptome analysis of Enterococcus faecalis toward its adaption to surviving in the mouse intestinal tract**. *Archives of microbiology* 2014, **196**(6):423-433.

28. Kristich CJ, Manias DA, Dunny GM: **Development of a method for markerless genetic exchange in Enterococcus faecalis and its use in construction of a srtA mutant**. *Applied and environmental microbiology* 2005, **71**(10):5837-5849.

CLAIMS

We claim

1. A modified Enterococcal strain, wherein the strain comprises:
   a. a ΔsrtA mutation,
   b. a first plasmid encoding Bac 21, wherein the plasmid has a Δ*bacAB* mutation, and
   c. a second plasmid encoding a proximal part of *bac-21operon*.

2. The modified strain on claim 1, wherein the strain is either *Enterococcus faecalis* or *Enterococcus faecium.*

3. The modified strain of claim 1, wherein the proximal segment is *bacABCDE*.

4. The modified strain of claim 1, wherein the strain is inefficient at establishing long-term colonization of the GI tract compared to its respective isogenic wild- type, but remains capable of delivering bacteriocin efficiently to decolonize antibiotic-resistant enterococci to the levels below the limit of detection (typically 100 CFU ml^{-1}) .

5. The modified strain of claim 4, additionally comprising a minimized potential for transfer of bacteriocin-production traits to other bacteria via conjugation.

6. The modified strain of any one of the preceding claims, wherein the strain comprises ΔsrtA mutation of SEQ ID NO:9.

7. The modified strain of any one of the preceding claims, wherein the first plasmid comprises SEQ ID NO:1.

8. The modified strain of any one of the preceding claims, wherein the second plasmid is SEQ ID NO:4.

9. A composition comprising the modified strain of any one of the preceding claims and a pharmaceutically acceptable carrier.

10. An animal feed composition comprising the modified strain of any one of claims 1-8 and at least one animal feed product.

11. A method of creating a modified Enterococcal strain, comprising the steps of:
a. obtaining an Enterococcal strain with a ΔsrtA mutation, and
b. introducing a first and a second plasmid into the strain, wherein the first plasmid encodes Bac21, wherein the first plasmid is a Δ*bacAB* mutant, and wherein the second plasmid encodes a segment proximal to the *bacA* promoter.

12. The method of claim 11, wherein the Enterococcal strain is either *Enterococcus faecalis* or *Enterococcus faecium.*

13. The method of claim 11 or 12, wherein the ΔsrtA mutation is a deletion of SEQ ID NO:8 from the open reading frame of *srtA.*

14. The method of any one of claims 11-13, wherein the first plasmid comprises SEQ ID NO:1.

15. The method of any one of claims 11-14, wherein the second plasmid comprises SEQ ID NO:4.

16. A modified enterococcal strain made by the method of any one of claims 11-15.

17. A method of treating enterococci infections in a human patient in need thereof, the method comprising: administering to the patient a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9.

18. A method of inhibiting the GI colonization by antibiotic-resistant enterococci in human patient in need thereof, the method comprising: administering to the patient a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9.

19. A method of altering the microbial composition of the GI track of a human patient, the method comprising: administering to the patient a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9.

20. A method of preventing GI track colonization of antibiotic resistant enterococci in a human patient, the method comprising administering to the patient a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9.

21. The method of any one of claims 17-20, wherein the modified enterococcal strain is administered orally.

22. The method of claim any one of claim 17-20, the method first comprising detecting antibiotic resistant enterococcal bacteria in the GI track of the human patient.

23. A method of treating enterococci infections in a non-human animal, the method comprising administering to the non-human animal a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9 or 10.

24. The method of claim 23, wherein the modified enterococcal strain is administered in the animal food or water.

25. A method of preventing GI track colonization of antibiotic resistant enterococci in a non-human animal, the method comprising: administering to the non-human

animal a therapeutically effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of claim 9 or 10.

26. The method of any one of claims 23-25, wherein the non-human animal is a poultry.

27. The method of claim 26, wherein the poultry is a chicken, turkey, quail or duck.

28. The method of any one of claims 23-25, wherein the non-human animal is a livestock animal.

29. The method of any one of claims 23-25, wherein the non-human animal is a domestic animal.

30. The method of claim 29, wherein the domestic animal is a cat, dog, pig, or bird.

31. A method of reducing contamination of livestock food products with antibiotic-resistant enterococci, the method comprising administering to the livestock before slaughter an effective amount of the modified enterococcal strain of any one of claims 1-8 or the composition of 9 or 10 in an amount effective to reduce the amount of antibiotic-resistant enterococci in the GI track of the livestock.

32. The method of claim 31, wherein the livestock is a poultry.

33. The method of claim 32, wherein the poultry is a chicken.

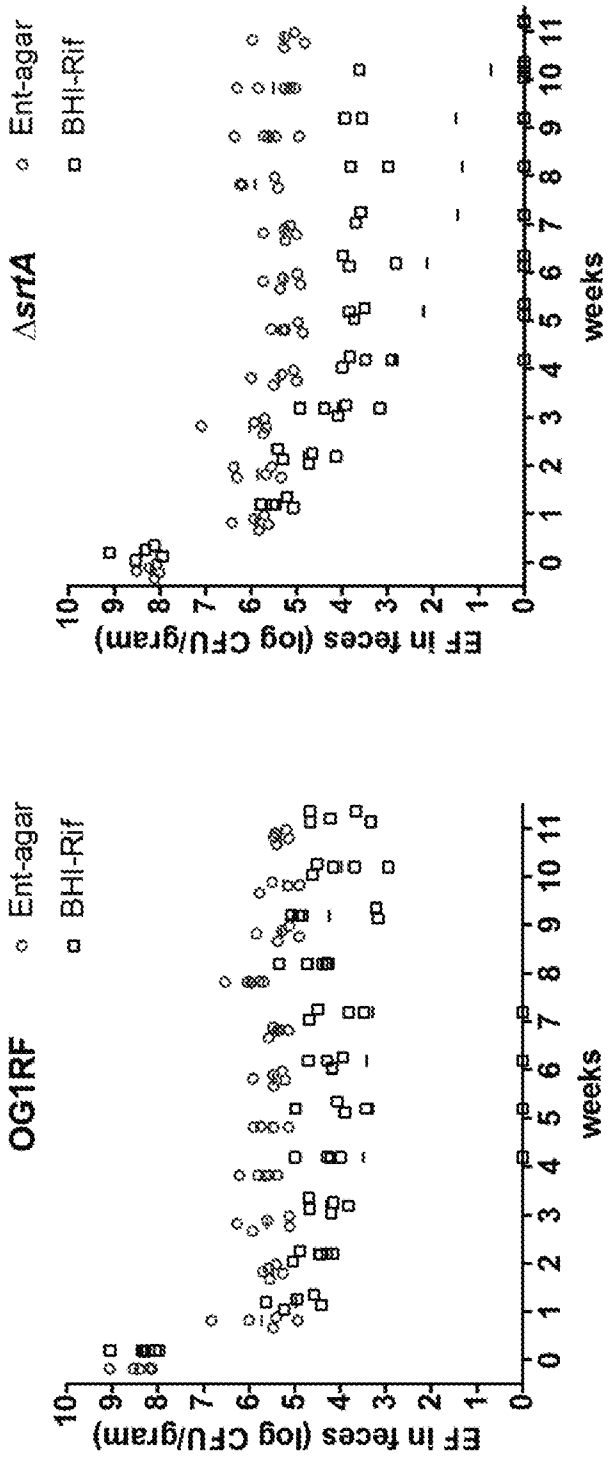

ΔsrtA
Ent-agar
BHI-Rif
EF in feces (log CFU/gram)
weeks
OG1RF
Ent-agar
BHI-Rif
EF in feces (log CFU/gram)
weeks

Fig. 1A

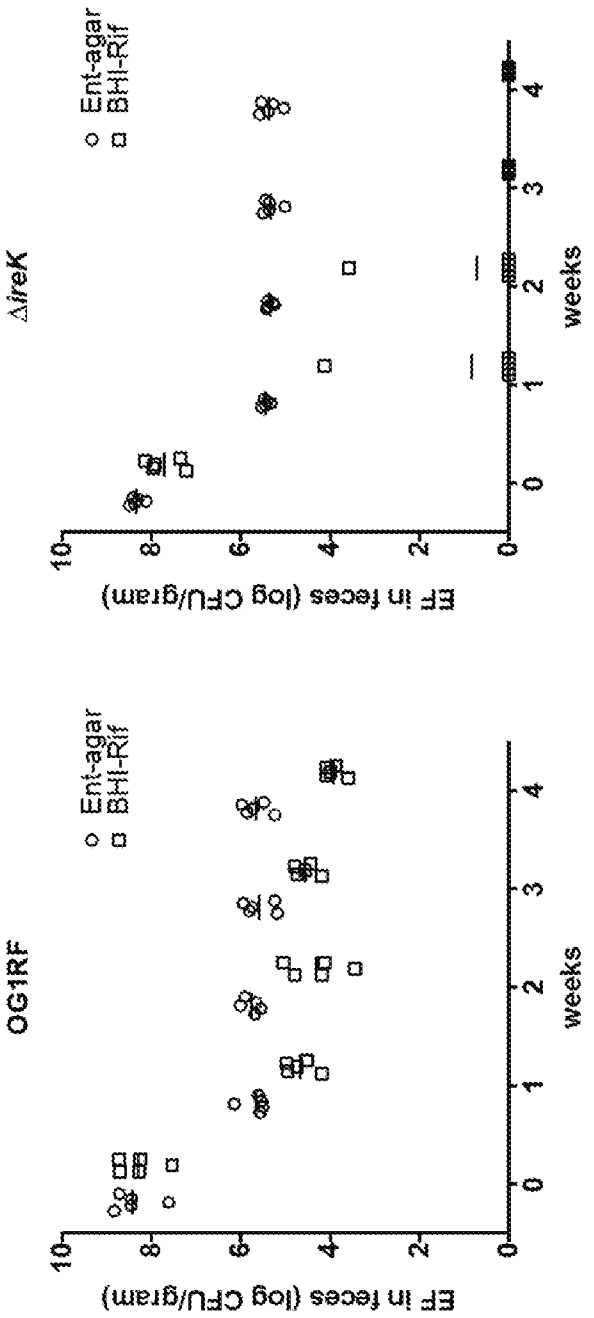

ΔireK
Ent-agar
BHI-Rif
EF in feces (log CFU/gram)
weeks
OG1RF
Ent-agar
BHI-Rif
EF in feces (log CFU/gram)
weeks

Fig. 1B

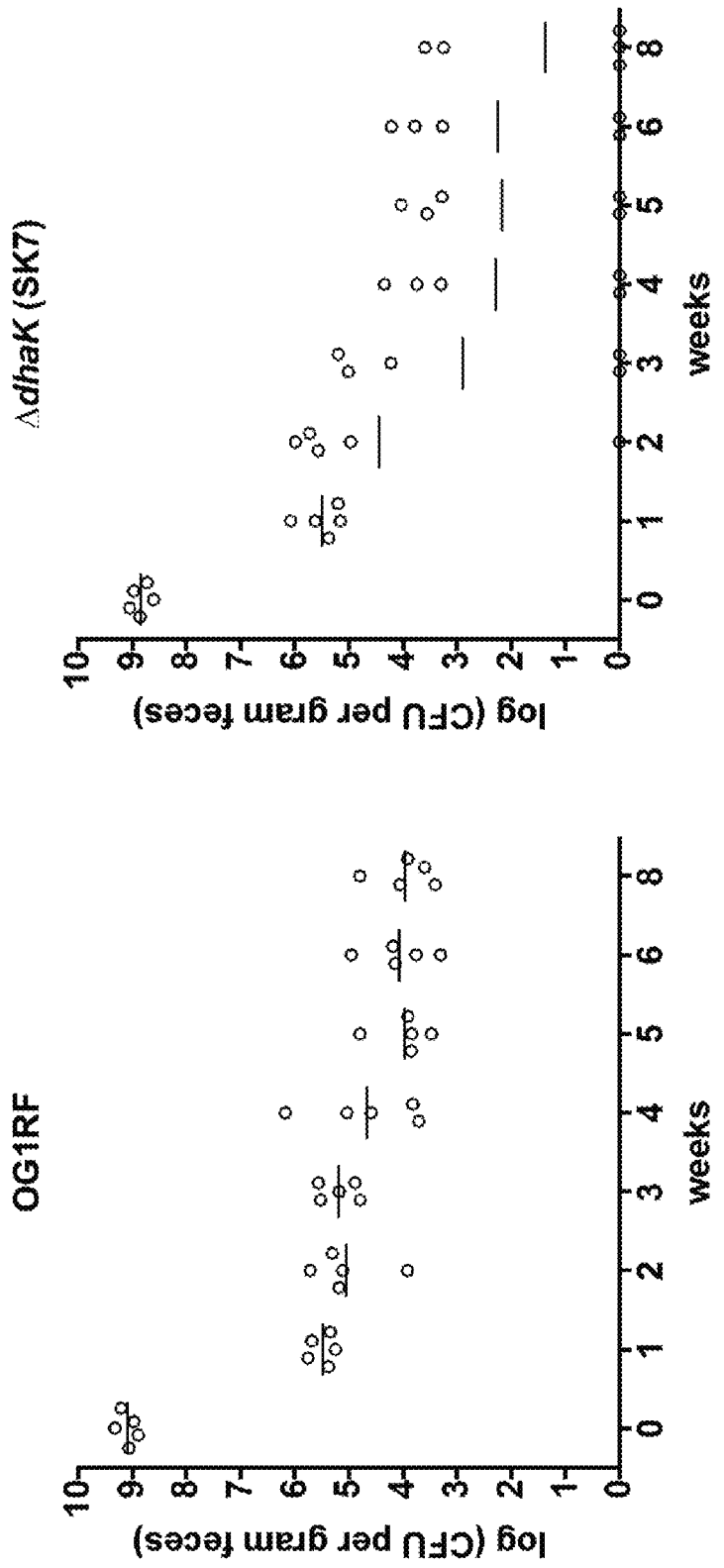


Fig. 1C

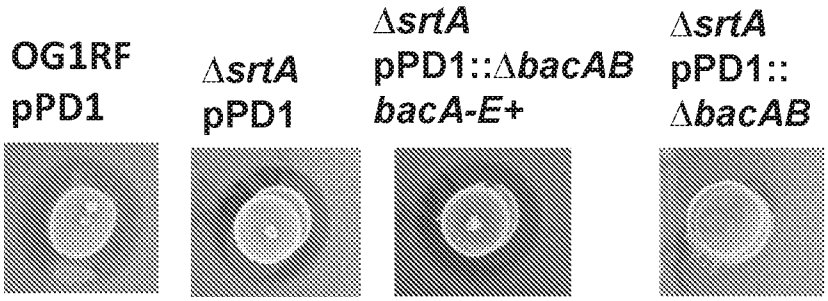


Fig. 2A

Recipient	Donor	Transconjugants after 24 hours
OG1SP	OG1RF pPD1	*25/25
OG1SP	Δ*srtA* pPD1	*3/50
OG1SP	Δ*srtA* pPD1::Δ*bacAB*, bacABCDE+	**0/50
OG1SP	Δ*srtA* pPD1::Δ*bacAB*	**0/25

Fig. 2B

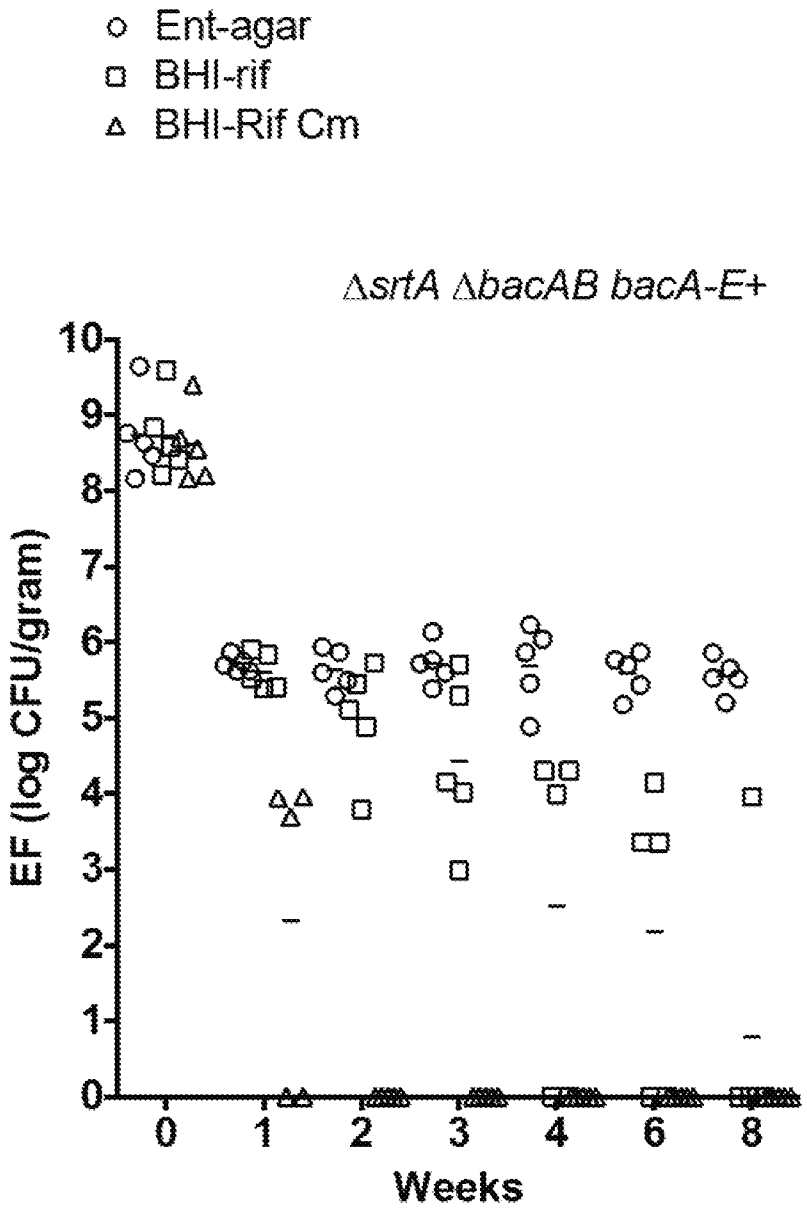
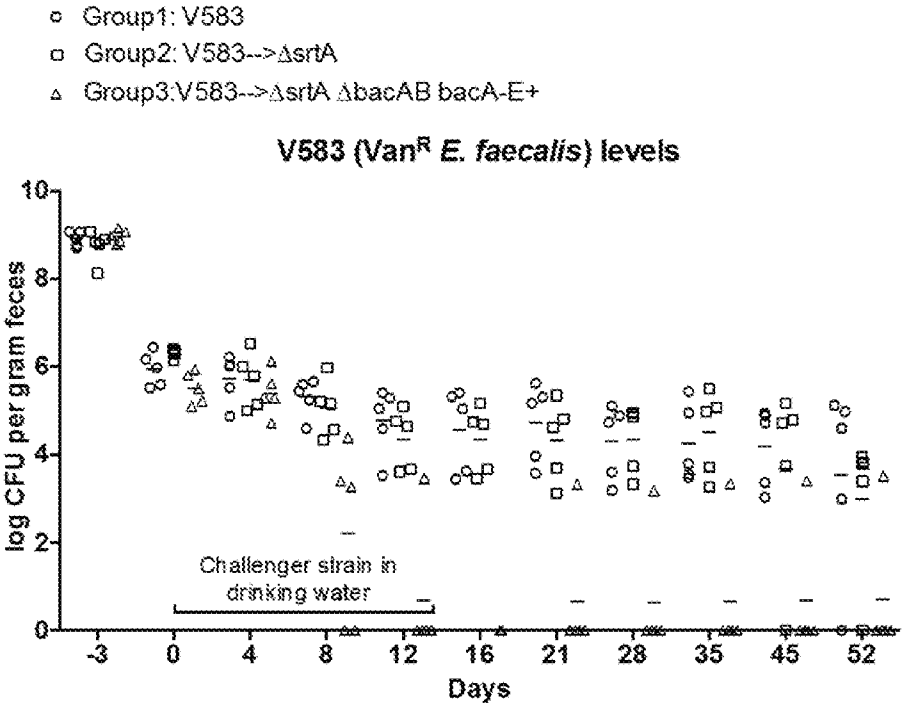

Ent-agar
BHI-rif
BHI-Rif Cm
ΔsrtA ΔbacAB bacA-E+
EF (log CFU/gram)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
6
8
Weeks

Fig. 3

A


Group1: V583
Group2: V583-->ΔsrtA
Group3:V583-->ΔsrtA ΔbacAB bacA-E+
V583 (VanR E. faecalis) levels
log CFU per gram feces
Challenger strain in drinking water
Days

B

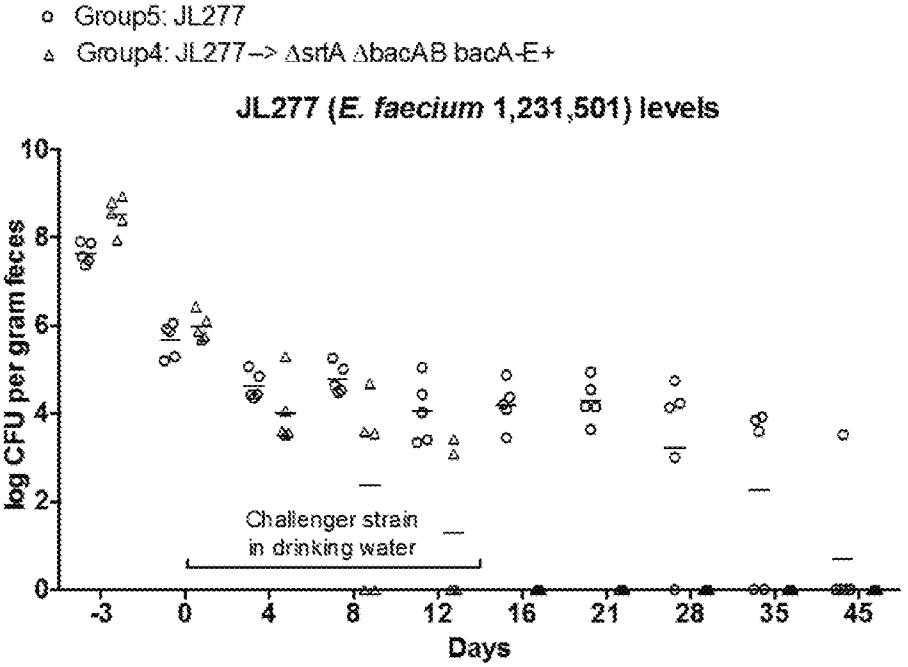

Group5: JL277
Group4: JL277--> ΔsrtA ΔbacAB bacA-E+
JL277 (E. faecium 1,231,501) levels
log CFU per gram feces
Challenger strain in drinking water
Days

Fig. 4A-4B

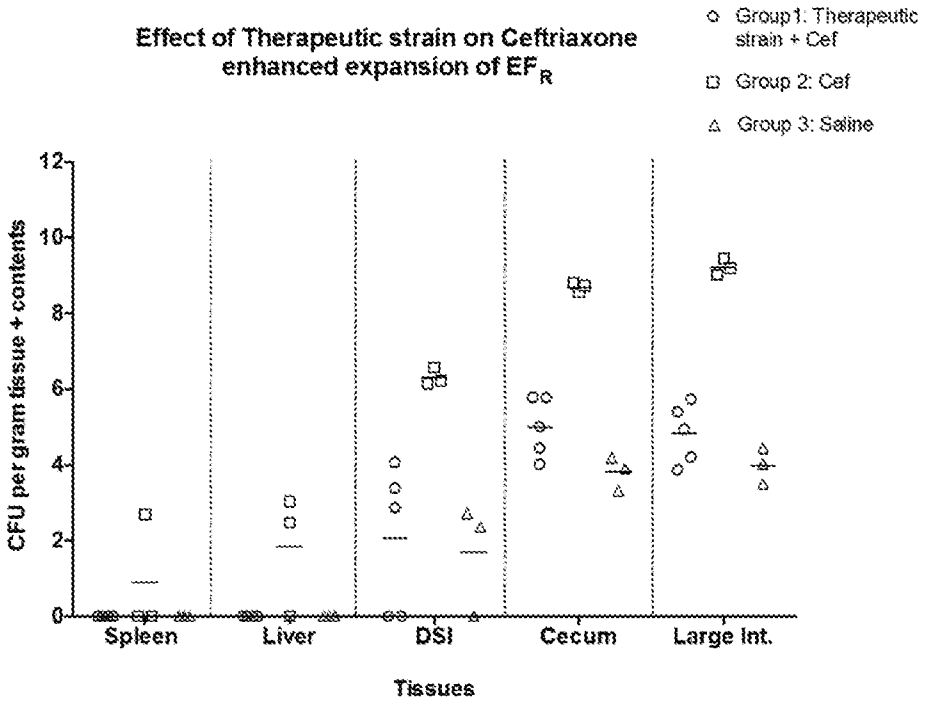

Effect of Therapeutic strain on Ceftriaxone enhanced expansion of EF$_R$
Group 1: Therapeutic strain + Cef
Group 2: Cef
Group 3: Saline
CFU per gram tissue + contents
0
2
4
6
8
10
12
Spleen
Liver
DSI
Cecum
Large Int.
Tissues

Fig. 5

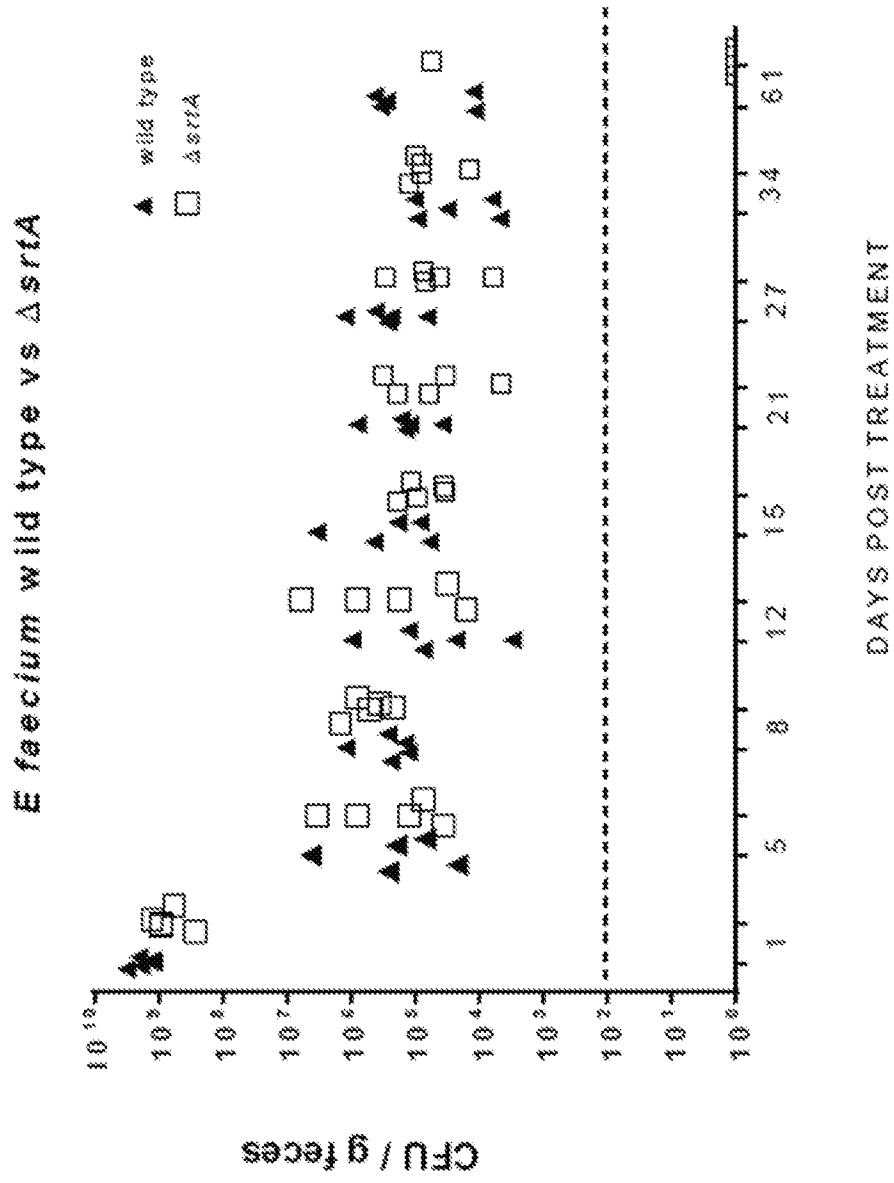

E faecium wild type vs ΔsrtA
wild type
ΔsrtA
CFU / g feces
DAYS POST TREATMENT

Fig. 6

Fig. 7

Deletion of *bacAB* in pPD1

Partial pPD1 Δ*bacAB*::*ermC* (SEQ ID NO:1, contains part of pPD1 backbone and including the *ermC gene* insert, full pD1 sequence found in SEQ ID NO:10)

Sequence of *ermC* insert in red (replaces *bacAB*) (SEQ ID NO:2, underlined and bold)

SEQ ID NO:3 (fragment of backbone PD1 (full pPD1 found in SEQ ID NO10):, non-bold, non-underline)

ttccagagaatcggatggtattaatttaaagaacaatcagtattctgaaaaacaaataaatgttctcgatcagt
tacaaaagttagattttaagattgaggaattagaaagtaccttacagatagaagatgagaggttcaatctggaag
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ccaaagatattatggtgtttgacaggttatctttagaatttt

Fig. 8

pSK29 -- Plasmid for expression of BacA-E (in pAM401 vector) (SEQ ID NO:4)

Sequence in **black**: pAM401 vector (SEQ ID NO:5)
Sequence in Red: *bacA-E* nucleotide sequence (SEQ ID NO:6)-bold and underlined

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TGAACGGTTTTCGTCTGCATACGTACCATAACGATAAACGTTTGGTGTCCAATGCGTAAATGTATCTTGATTTTCGTGAATCGC
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Fig. 8 (continued)

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AGAGAAGAAATGTACCCTTATCTATCTTTTGGATCAGAAAGGAATCAATTTACGCTTCTAAGATCGTCTCCGAATGGTTTGAAT
AAAGTTTTTAATTCGAAACTAACGATATACAGGTTATTTTTATTAATTCCTTTACTTATATTAATTATCATAAATATTGTAGTT
TCTGTATACATTATGATTCCGGTAATTTTTGCTATCTTTTTGTTTATAACATTTTCTATGTCTGTGTATGTTTTCCCTATGATT
CAAATGTATATGATTCCTTTAGCTACTAAACTAGATTACACTAATGATACAGAAATTGGAAGTGCTAAAGATGAAAAAATTGTT
TTAGAGAAATTTCAAACAGTTCCAAGATACTTTTTAATATCGTTCCTTTAGTGCTTACATTTATCTTTCCAATTGTAGGGGAA
AGTTATTCATTAATAATATTTTTCGGAGAGTTAGTATATTTCTCTTTAGCAACAATAATATTTGTGTTTTTCAGTAAAAAAATT
ATTAGGAAAGGAATTTTTGTTGTACATGCGATTAGATATCATTAATTTTGAGAAATATTTGAAAATTTCCTTCTATATATCAAT
ATTTATTTTTTTTTCTGGGAGTGTTAGCAGGGATTATAATAGGTCCTCATATAAATAAATTGGACTATTTTGGTCAGGAAGTTTC
ATTTTATAGTGTTAGTGTTAATAATTTAAAGGTCTCTTTTTATTTCCTCACTATAGGAATGGTAACAGGGGGGATTTATGCATT
TTTATTTATGGGTATAAATGGTTATATAATTGGTAAGTTGATTCAATATTTATACATTAACAATGAACTAAATGTTTTGTATAA
AGGTCTTCTTCCACATTTTTTTATAGAGCTTTTAGGATTGGCAACATTTAGTATGATAAGTACCATTCCAATATTTGTTATTTT
GGTTTTTTTTTAAAACGCCTACACATGTAGTTCCTATTAAAAAAATCATAAAGTTAAGTGTGTTTTTTACTGTACTTGGAATAGT
TTTAATAATAATTGGAGGATATATAGAATCTAATATAAGCTATGTAGATATACGATAGAAGTTGAGATAGAAAACTAATCTCAA
AGGAAGATATCCGCCTATTTCCAAACGTTATACGAAAGAATTTAAACAAACCATTGATCACGAATCAAAAATGCTGAATTAAAG
ATATACTCATTAAAGGAAGAGTGGGCACTATTTTAAATTTATACAACCAAGGTAAAAATTTTCGTGAATTATCTTTATAATAAT
ATGTCGGTTACTTAAAATTTTTGAAATTCACAATAAACATATAATTAACAGTGTGATAATTTATTTGTCTACAAGTCAATTGGG
ATAGGCAACTCTAGTTGCATAAAATATCTCTCACAAAAAGTGTTTCATGTATTACTGAATCTGATCCATATAAGTTCTTAAGAA
AGGAAGATAAGTTTGTACAAGTTTACTTTGAAAAAATACATCGGTATTACTTCACTATTTTTTTGTTTTCAAATATTCTAATT
GCTGAAGAGTTTATTTTTGTAGAAAAAAACTTGAGTTTTTTCCCTAAATTAGGGATAATTGTGCTTGTTTTTTTTATGAGTGCT
CCCGCTTTTGTTGTTGTTACTTTAATTTTGACTTGTTTAGTTGCATTTCTTATTAGTTTAATTAACAAATTTTACAGTTTCAAA
AGAGTATACTTATCGACTTTATTTGTAAATAGTATTCAGTTATTCGTAAATTTAGCTTTATTTAGTGTATTTTTACATTACAAT
TTAAATTTGCGATTGCTAAGTATAATGAGTTTTATGATTAATATTTTTTTAGTAGTTATCTATCGCAATTTATTAATTAAATTT
GCGAATGTAAACAGTAGGGCTGCGAATGTATTATCAATTTTCGGGATTGCTTTATCTGTAATTTATTTGGTGGTAGGAGTAAAG
TAATGAAAAAAATAACAATTAACAACTTGAGTTTTTATTATGAATCCAAAGATATTATGGTGTTTGACAGGTTATCTTTAGAAT
TTTCTTCTGAAAAAAGTTATGCACTAGTTGGTTCTAATGGTGTAGGAAAACAACATTGTTAAATATTTTATCAGGTATATATC
AACCCACAGGGGGAACAATAGAATATGACAGCACTTTGTATACAGAAAAAGTAACTAAAGAAAAAGTAGCTTTTATACCATATA

Fig. 8 (continued)

AAACTAAGCTATATCCTTATCTTGATGTTTTTGATCATATAAAGCTAATAGCAGAATTATGGGGAATTAAAACAGACTATTTAG
AATATAAAAGAAAAGTACTAGAATATTGTAGCCGTCTAAACTTGGACTATTATAATAAGAAAGTAGAGTCTTACTCTACAGGTA
TGGAGTATAAACTATACATTTCTTTAATGTTGGCAAGAGATGTTTCTCTTGTATTATTAGATGAACCTTTTACAATGTTAGATA
AAAAAAGTCGATATTTAGCTATGGACTTAATCAAAGAGAAAAAAATAATAACAATATTTTCTTCACATCAGAAAGATATTGTAG
AATATTTGTCAAATGATATTATTAATCTTGACAAACTGAAGGGAGTAAACTTGGAAAATTATGAAAAGAATTGATTACATTATT
ATTATAGTCTCACTATTAGCAACAATAGTCGCAATATTTTTAATAGGGATAGATTCTATGTTAGGCACAGGACGGGTGTGGTCG
CCATGATCGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGTGCTCCGA
GAACGGGTGCGCATAGAAATTGCATCAACGCATATAGCGCTAGCAGCACGCCATAGTGACTGGCGATGCTGTCGGAATGGACGA
TATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAGCCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGA
GCGCATTGTTAGATTTCATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTATCGATGA
TAAGCTGTCAAACATGAGAATTACAACTTATATCGTATGGGGCTGACTTCAGGTGCTACATTTGAAGAGATAAATTGCACTGAA
ATCTAGAAATATTTTATCTGATTAATAAGATGATCTTCTTGAGATCGTTTTGGTCTGCGCGTAATCTCTTGCTCTGAAAACGAA
AAAACCGCCTTGCAGGGCGGTTTTTCGAAGGTTCTCTGAGCTACCAACTCTTTGAACCGAGGTAACTGGCTTGGAGGAGCGCAG
TCACCAAAACTTGTCCTTTCAGTTAGCCTTAACCGGCGCATGACTTCAAGACTAACTCCTCTAAATCAATTACCAGTGGCTGC
TGCCAGTGGTGCTTTTGCATGTCTTTCCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGACTGAACGGG
GGGTTCGTGCATACAGTCCAGCTTGGAGCGAACTGCCTACCCGGAACTGAGTGTCAGGCGTGGAATGAGACAAACGCGGCCATA

ACAGCGGAATGACACCGGTAAACCGAAAGGCAGGAACAGGAGAGCGCACGAGGGAGCCGCCAGGGGGAAACGCCTGGTATCTTT
ATAGTCCTGTCGGGTTTCGCCACCACTGATTTGAGCGTCAGATTTCGTGATGCTTGTCAGGGGGGCGGAGCCTATGGAAAAACG
GCTTTGCCGCGGCCCTCTCACTTCCCTGTTAAGTATCTTCCTGGCATCTTCCAGGAAATCTCCGCCCCGTTCGTAAGCCATTTC
CGCTCGCCGCAGTCGAACGACCGAGCGTAGCGAGTCAGTGAGCGAGGAAGCGGAATATATCCTGTATCACATATTCTGCTGACG
CACCGGTGCAGCCTTTTTTCTCCTGCCACATGAAGCACTTCACTGACACCCTCATCAGTGCCAACATAGTAAGCCAGTATACAC
TCCGCTAGCGCTGATGTCCGGCGGTGCTTTTGCCGTTACGCACCACCCCGTCAGTAGCTGAACAGGAGGGACAGCTGATAGAAA
CAGAAGCCACTGGAGCACCTCAAAAACACCATCATACACTAAATCAGTAAGTTGGCAGCATCACCCGACGCACTTTGCGCCGAA
TAAATACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTT
TGGCGAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTT
GAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGG
CATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTT
TTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAA
TTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAA
ACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGT
GAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTT
GATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTG
ATGCCGCTGGCGATTCAGGTTCATCATGCCGTCTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGC
GATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTGCTACGCCTGAATAAGTGATA
ATAAGCGGATGAATGGCAGAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCAGGGCAGGGTCGTTAAATAGCCGCTTATGT
CTATTGCTGGTTTACCGGTTTATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGAGGCCAGTTTGCTCAGGC
TCTCCCCGTGGAGGTAATAATTGACGATATGATCATTTATTCTGCCTCCCAGAGCCTGATAAAAACGGTTAGCGGTTCGTTAAT
ACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTTGTTTCGGC
GTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACA
GTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTACCGGACAGCGGTGCGGACTGTTGTAACTCAGAA
TAAGAAATGAGGCCGCTCATGGCGTTGACTCTCAGTCATAGTATCGTGGTATCACCGGTTGGTTCCACTCTCTGTTGCGGGCAA
CTTCAGCAGCACGTAGGGGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTC
GCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAG
CCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGCCAGGACCCAACGCTGCCCGACTTTAAACGTGGATCA
TTTTCTTTAAATTTATGCTGACGACCTTTGAATTTGCCTTTTTTTCTTAGCAATTTCGATTCCTTGTGCCTGACGTTCCTTAATT
TTTTTTCGTTCTGATTCTGCTTGATACTTGTACAATTCAATGACAAGGCTATTAATCAAACGCCTTAAATTTTCATCTTCAATA
CCATTCATTGAGGGTAAATTTAAGACTTCCAGGGTTGCCCCCTTAATTTGAATTTGATTCATCAATTCTGTTAATTCTTTATTA
TTTCGTCCTAATCGATCTAATTCAGTAACAATAACAATATCCCCTTCACGAATATAGTTAAGCATAGCTTGTAATTGTGGGCGT
TCGACCGATTGACCGCTTAATTTGTCTGAAAAGACCTTAGAAACGCCCTGTAACGCTTGTAATTGCCGATCTAAGTTCTGTTCT
TTGCTACTGACACGTGCATAACCAATTTTAGCCATTTTCAACCAACCTCTAAAATTCTCTCGGTTGCAATAACCAATCAGCAAT
ATCTACTTTTTCAATTTCAAATTGCTTATCAGAAATTGTCTTTTCGTAAGCGATAAAATCTTGCGCATATTGTTGCTCATTAAA
AATAGCCACCACTTCGTCATTTTCTAAAACTCGATAAATAAATTTTTTCATTTTACTCCTCCTATTATGCCCAACTTAAATGAC
CTATTCACCAAGTCAATTATACTGCTAAAATCATATTAGGACAAATAGGTATACTCTATTGACCTATAAATGATAGCAACTTAA
AAGATCAAGTGTTCGCTTCGCTCTCACTGCCCCTCGACGTTTTAGTAGCCTTTCCCTCACTTCGTTCAGTCCAAGCCAACTAAA
AGTTTTCGGGCTACTCTCTCCTTCTCCCCCTAATAATTAATTAAAATCTTACTCTGTATATTTCTGCTAATCATTCACTAAACA
GCAAAGAAAAACAAACACGTATCATAGATATAAATGTAATGGCATAGTGCGGGTTTTATTTTCAGCCTGTATCGTAGCTAAACA
AATCGAGTTGTGGGTCCGTTTTGGGGCGTTCTGCCAATTTGTTTAGAGTTTCTTGAATAAATGTACGTTCTAAATTAAACGAAG
CTGTCAGCGCCTTTATATAGCTTTCTCGTTCTTCTTTTTTTAATTTAATGATCGATAGCAACAATGATTTAACACTAGCAAGTT
GAATGCCACCATTTCTTCCTGGTTTAATCTTAAAGAAAATTTCCTGATTCGCCTTCAGTACCTTCAGCAATTTATCTAATGTCC
GTTCAGGAATGCCTAGCACTTCTCTAATCTCTTTTTTGGTCGTCGCTAAATAAGGCTTGTATACATCGCTTTTTTCGCTAATAT
AAGCCATTAAATCTTCTTTCCATTCTGACAAATGAACACGTTGACGTTCGCTTCTTTTTTTCTTGAATTTAAACCACCCTTGAC
GGACAAATAAATCTTTACTGGTTAAATCACTTGATACCCAAGCTTTGCAAAGAATGGTAATGTATTCCCTATTAGCCCCTTGAT
AGTTTTCTGAATAGGCACTTCTAACAATTTTGATTACTTCTTTTTCTTCTAAGGGTTGATCTAATCGATTATTAAACTCAAACA

Fig. 8 (continued)

TATTATATTCGCACGTTTCGATTGAATAGCCTGAACTAAAGTAGGCTAAAGAGAGGGTAAACATAACGCTATTGCGCCCTACTA
AACCCTTTTCTCCTGAAAATTTCGTTTCGTGCAATAAGAGATTAAACCAGGGTTCATCTACTTGTTTTTTGCCTTCTGTACCGC
TTAAAACCGTTAGACTTGAACGAGTAAAGCCCTTATTATCTGTTTGTTTGAAAGACCAATCTTGCCATTCTTTGAAAGAATAAC
GGTAATTGGGATCAAAAAAATTCTACATTGTCCGTTCTTGGTATACGAGCAATCCCAAAATGATTGCACGTTAGATCAACTGGCA
AAGACTTTCCAAAATATTCTCGGATATTTTGCGAGATTATTTTGGCTGCTTTGACAGATTTAAATTCTGATTTTGAAGTCACAT
AGACTGGCGTTTCTAAAACAAAATATGCTTGATAACCTTTATCAGATTTGATAATTAACGTAGGCATAAAACCTAAATCAATAG
CTGTTGTTAAAATATCGCTTGCTGAAAT

Fig. 9

Sortase A deletion

E. faecalis OG1RF *srtA* open reading frame (SEQ ID NO:7)(the sequence deleted in Δ*srtA* is indicated in red, bold and underlined (SEQ ID NO:8):, SEQ ID NO:9 is the OG1RFΔsrtA (black sequence, non-underline, non-bold)

ATGCGCCCAAAAGAGAAAAAAAGAGGAAAAAATTGGTTAATCAACAGTTTATTAGTTTTACTATTTATCATTGGC
TTGGCCTTAATTTTTAACAATCAGATACGCAGTTGGGTGGTTCAACAAAATAGCCGCTCGTACGCCGTTAGCAAG
TTGAAACCAGCCGATGTGAAGAAAAATATGGCTCGTGAAACAACGTTTGACTTTGATTCAGTTGAGTCCTTGAGC
ACAGAAGCGGTGATGAAAGCCCAATTTGAAAACAAAAACTTACCTGTGATTGGTGCCATTGCGATACCAAGTGTC
GAAATTAATTTGCCCATTTTTAAAGGATTGTCAAATGTCGCTTTATTAACTGGTGCCGGGACCATGAAAGAAGAT
CAAGTCATGGGGAAAAACAATTATGCCTTGGCTAGTCATCGAACGGAAGATGGCGTTTCCTTATTTTCACCTTTA
GAAAGAACCAAAAAAGACGAACTCATTTATATCACTGATTTATCTACTGTTTATACATACAAAATAACTTCTGTA
GAAAAAATCGAACCAACCCGTGTTGAGTTAATTGATGACGTTCCTGGTCAAAATATGATTACCTTAATTACCTGT
GGCGATTTACAAGCAACGACGCGAATTGCTGTTCAAGGAACATTAGCAGCAACGACGCCTATTAAAGACGCCAAC
GACGATATGTTAAAGGCTTTCCAATTGGAGCAAAAAACCTTAGCCGATTGGGTGGCTTAA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/18099

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 39/085; A61P 31/04; C12N 1/21, 15/31; C12R 1/01, 1/44, 1/46 (2018.01)

CPC - A61K 39/085; A61P 31/04; C07K 14/31; C12N 1/36; C12R 1/01, 1/44, 1/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/0236419 A1 (SCHNEEWIND et al.) September 12, 2013; abstract; paragraphs [0015], [0080], [0087], [0168], [0178]	1-5, 6/1-5, 9-10, 11/9-10
A	US 2008/0219960 A1 (GROOT et al.) September 11, 2008; paragraph [0037]	1-5, 6/1-5, 9-10, 11/9-10
A	US 5,032,512 A (WITHOLT et al.) July 16, 1991; column 2, lines 67-68	1-5, 6/1-5, 9-10, 11/9-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 April 2018 (12.04.2018)	26 APR 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/18099

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-8, 9A, 10A, and 12-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.