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(71) Applicant: ELAINE TSIUMAS KAZNESSIS [US/US];
330 Wyndam Circle West, New Brighton, MN 55112 (US).

(72) Inventor: KAZNESSIS, Yiannis, N.; 1000 Westgate Drive,
Suite 1007, St. Paul, MN 55114-2010 (US).

(74) Agent: NOLAND, Greta, E.; MARSHALL, GERSTEIN
& BORUN LLP, 233 S. Wacker Drive, 6300 Willis Tower,
Chicago, IL 60606-6357 (US).

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(54) Title: PERSONALIZED BACTERIAL CANCER VACCINES

(57) Abstract: The disclosure provides Personalized Bacterial Cancer Vaccines (PBCVs) and related methods, including methods of isolation of bacteria from a patient's gut microbiome, genetic engineering of selected bacteria, administration routes and dosing, manufacturing, regulatory approval, validation tests, and assessment of immune response and treatment efficacy.

WO 2025/024603 A2

PERSONALIZED BACTERIAL CANCER VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/515,419, filed July 25, 2023, which is incorporated herein by reference in its entirety.

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

[0002] This application contains, as a separate part of disclosure, a Sequence Listing in computer-readable form (Filename: 59305_SeqListing.xml; 60,976 bytes – XML file created July 24, 2024) which is incorporated by reference herein in its entirety.

FIELD

[0003] This invention presents Personalized Bacterial Cancer Vaccines (PBCVs) and related methods, a novel approach to cancer treatment. A method involves collecting normal and cancer tissue samples from a patient, sequencing the samples to identify DNA sequence differences, discovering and selecting unique, patient-specific neoantigens. This method includes isolating bacteria from patient stool samples, characterizing these bacteria, selecting ones that can safely recolonize the patient's intestinal tract. The method involves genetically engineering the selected bacterial isolates to express and secrete the discovered and selected neoantigens, thus creating a PBCV specific to the patient and their cancer. The method includes administering the recombinant PBCV orally or rectally, the PBCVs colonizing the gastrointestinal tract and releasing neoantigens inside the gastrointestinal tract to interact with the patient's immune system. The patient's immune system responds by mounting an attack against cancer cells. This highly personalized and targeted approach enhances immunogenicity while differentiating cancer from healthy cells and allowing for local and systemic cancer cell killing. The PBCVs are safe and effective against diverse cancer types, including solid cancers, hematological cancers and pediatric cancers, and can be engineered to stay ahead of cancer cell mutations.

BACKGROUND

[0004] Cancer, characterized by uncontrolled cell growth and proliferation, remains a global health challenge with significant morbidity and mortality rates. Conventional cancer treatment modalities, such as surgery, chemotherapy, and radiation therapy, often exhibit limitations, including adverse side effects and incomplete eradication of tumors. Therefore, alternative therapeutic strategies are being explored to harness the power of the immune system for precise and effective cancer management.

[0005] In recent years, cancer vaccines have emerged as a promising avenue in the field of immunotherapy. By stimulating the patient's immune system to recognize and attack cancer cells, vaccines have the potential to achieve targeted tumor destruction and long-lasting immune memory. However, the development of cancer vaccines faces inherent complexities due to the heterogeneity and adaptability of cancer cells.

BRIEF SUMMARY

[0006] Personalized Bacterial Cancer Vaccines (PBCVs) represent an innovative strategy that combines the unique characteristics of an individual's cancer with components of the patient's microbiome and with the immunogenic potential of genetically engineered bacteria releasing neoantigens inside the intestinal tract of the patient. This approach (schematically depicted in Figure 1) aims to elicit a robust and specific immune response against cancer cells while minimizing off-target effects.

[0007] The personalized nature of PBCVs offers several distinct advantages. First, by utilizing patient-specific neoantigens, which are derived from genetic alterations found in the patient's cancer cells, PBCVs can potentially overcome the limitations of traditional vaccines that target shared antigens. This approach ensures that the vaccine precisely matches the patient's tumor profile, optimizing its immunogenicity and therapeutic potential.

[0008] Second, by utilizing non-pathogenic bacteria selected from the patient's existing microbiome, PBCVs can be orally or rectally administered, reach and safely colonize the gastrointestinal tract of the patient. The bacteria that become the organism basis for PBCVs can be selected for a) non-pathogenicity and non-virulence, b) survival through the stomach of the patient, and c) metabolic activity inside the GI tract. These bacteria can also be amenable to genetic modifications and be amenable to manufacturing.

[0009] Third, PBCVs capitalize on the vast immune system residing in the gastrointestinal (GI) tract. The administration of PBCVs orally or rectally allows for targeted delivery to the GI tract, where a significant proportion of the immune system is localized. This targeting strategy takes advantage of the intricate interactions between the gut and the immune system, augmenting the immune response against cancer cells expressing neoantigens.

[0010] Furthermore, the use of non-pathogenic bacteria, such as probiotics, as delivery vehicles for PBCVs offers an added layer of safety. These bacteria are inherently designed to interact with the gut environment and are well-tolerated by the human host. Their ability to colonize the GI tract and release neoantigens locally enhances the efficiency of antigen presentation and subsequent immune activation.

[0011] Overall, the personalized approach of PBCVs holds immense potential for treating, controlling, and preventing a wide range of cancers, including both hematological and solid tumors. This approach can be extended to pediatric cancers, offering new avenues for improved therapeutic options. By engineering the vaccines to be one step ahead of cancer cell mutations, PBCVs may address the challenges posed by tumor heterogeneity and the development of resistance.

[0012] In conclusion, personalized Bacterial Cancer Vaccines represent an innovative and promising strategy to harness the immune system's potential for precise and effective cancer management. The personalized nature, targeted delivery to the GI tract, and utilization of patient-specific neoantigens make PBCVs a compelling therapeutic approach for combating cancer, offering new hope in the battle against this devastating disease.

[0013] As used herein, “neoantigen” refers to a new protein that forms on cancer cells when certain mutations occur in tumor DNA. According to the US National Cancer Institute “Neoantigens may play an important role in helping the body make an immune response against cancer cells. Neoantigens used in vaccines and other types of immunotherapy are being studied in the treatment of many types of cancer.”

[0014] As used herein, the terms “peptide” and “protein” refers broadly to a polymer of two or more amino acids joined together by peptide bonds. The term “protein” also includes molecules which contain more than one protein joined by a disulfide bond, or complexes of proteins that are joined together, covalently or noncovalently, as multimers (for example, dimers, trimers, tetramers). Thus, the terms peptide, oligopeptide, enzyme, subunit, and protein are all included within the definition of protein and these terms are used interchangeably. It should be

understood that these terms do not connote a specific length of a polymer of amino acids, nor are they intended to imply or distinguish whether the protein is produced using recombinant techniques, chemical or enzymatic synthesis, or is naturally occurring.

[0015] As used herein, “neoantigen peptide vaccine” refers to a peptide-based cancer vaccine consisting of patient-specific antigens, which are immunogenic and unique to the patient's tumor, with potential immunomodulating and antineoplastic activities. According to the US National Cancer Institute “Upon vaccination with the neoantigen peptide vaccine, the peptides stimulate the host immune system to mount a specific cytotoxic T-lymphocyte (CTL) response against tumor cells expressing the neoantigens, which results in tumor cell lysis”.

[0016] As used herein, the term “polynucleotide” refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded RNA and DNA. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. A polynucleotide can be linear or circular in topology. A polynucleotide may be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment. A polynucleotide may include nucleotide sequences having different functions, including, for instance, coding regions, and non-coding regions such as regulatory regions.

[0017] As used herein, the terms “coding region,” “coding sequence,” and “open reading frame” are used interchangeably and refer to a nucleotide sequence that encodes a protein and, when placed under the control of appropriate regulatory sequences expresses the encoded protein. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A “regulatory sequence” is a nucleotide sequence that regulates expression of a coding sequence to which it is operably linked. Non-limiting examples of regulatory sequences include promoters, enhancers, transcription initiation sites, translation start sites, translation stop sites, and transcription terminators. The term “operably linked” refers to a juxtaposition of components such that they are in a relationship permitting them to function in their intended manner. A regulatory sequence is “operably linked” to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

[0018] As used herein, a “polycistronic mRNA” refers to a transcription product that includes two or more coding regions. Expression of the two or more coding regions is controlled by a single promoter, and the series of the two or more coding regions that are transcribed to produce a polycistronic mRNA is referred to as an operon.

[0019] As used herein, “probiotic” refers to live microorganisms which when administered in adequate amounts confer a health benefit on the host. This is the definition offered by the Food and Agriculture Organization of the United Nations and the World Health Organization.

[0020] As used herein, “genetically modified bacterium” refers to a bacterium that has been altered “by the hand of man.” A genetically modified bacterium includes a bacterium into which an exogenous polynucleotide, for example, an expression vector, has been introduced.

[0021] As used herein, a “vector” is a replicating polynucleotide, such as a plasmid, to which another polynucleotide may be attached so as to bring about the replication of the attached polynucleotide.

[0022] As used herein, an “exogenous protein” and “exogenous polynucleotide” refers to a protein and polynucleotide, respectively, which is not normally or naturally found in a microbe, and/or has been introduced into a microbe. An exogenous polynucleotide may be separate from the genomic DNA of a cell (for example, it may be a vector, such as a plasmid), or an exogenous polynucleotide may be integrated into the genomic DNA of a cell.

[0023] As used herein, a “heterologous” polynucleotide, such as a heterologous promoter, refers to a polynucleotide that is not normally or naturally found in nature operably linked to another polynucleotide, such as a coding region. As used herein, a “heterologous” protein or “heterologous” amino acid refers to amino acids that are not normally or naturally found in nature flanking an amino acid sequence.

[0024] Unless modified as otherwise described herein, a pair-wise comparison analysis of amino acid sequences can be carried out using the Blastp program of the BLAST 2 search algorithm, as described by Tatiana *et al.*, FEMS Microbiol. Lett., 1999; 174:247-250, and available on the National Center for Biotechnology Information (NCBI) website. The default values for all BLAST 2 search parameters may be used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. Alternatively, polypeptides may be compared using the BESTFIT algorithm in the GCG package (version 10.2, Madison WI).

[0025] In the comparison of two amino acid sequences, structural similarity may be referred to by percent “identity” or may be referred to by percent “similarity.” “Identity” refers to the presence of identical amino acids. “Similarity” refers to the presence of not only identical amino acids but also the presence of conservative substitutions.

[0026] Thus, as used herein, a candidate protein useful in the methods described herein includes those with at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% amino acid sequence similarity to a reference amino acid sequence.

[0027] Alternatively, as used herein, a candidate protein useful in the methods described herein includes those with at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% amino acid sequence identity to the reference amino acid sequence.

[0028] Conditions that are “suitable” for an event to occur, such as expression of an exogenous polynucleotide in a cell to produce a protein, or production of a product, or “suitable” conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event.

[0029] As used herein, an “animal” includes members of the class Mammalia and members of the class Aves, such as human, avian, bovine, caprine, ovine, porcine, equine, canine, and feline.

[0030] The term “and/or” means one or all of the listed elements or a combination of any two or more of the listed elements.

[0031] The words “preferred” and “preferably” refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

[0032] The terms “comprises” and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

[0033] Unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one.

[0034] Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (for example, 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

[0035] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

[0036] Reference throughout this specification to “one embodiment,” “an embodiment,” “certain embodiments,” or “some embodiments,” etc., means that a particular feature, configuration, composition, or characteristic described in connection with the embodiment is included in at least one embodiment of the disclosure. Thus, the appearances of such phrases in various places throughout this specification are not necessarily referring to the same embodiment of the disclosure. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more embodiments.

[0037] Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0038] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

[0039] All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

[0040] The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in

various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF DRAWINGS

[0041] Figure 1 is a schematic depiction of the process for selecting, developing, manufacturing and administering Personalized Bacterial Cancer Vaccines (PBCVs). Tissue and cell samples are collected from a cancer patient. The genomic material of tumor and normal cells is sequenced. The DNA/RNA sequence differences between the tumor and normal cells are determined. Patient-specific neoantigen peptides are discovered. Selected neoantigens are encoded in recombinant DNA. Bacteria are isolated from patient's stool samples and characterized. These bacteria or other probiotic bacteria are genetically engineered for the expression and secretion of the neoantigen peptides. The immunogenicity of secreted neoantigens is tested in laboratory in vitro and in vivo experiments. The genetically modified probiotic is manufactured and formulated for oral administration. The patient is administered PBCVs orally. Patient samples are collected to monitor the patient's immune responses against cancer.

[0042] Figure 2 is a schematic of plasmid pGP8001, used to engineer *Bacillus* spp. for the expression of cancer neoantigens. The plasmid contains the transcriptional units for thyA and a Tp53 neoantigen. In this case, the neoantigen is the p53 sequence from amino acid number 165 to amino acid 185, with a single mutation, R175H. In this case, thyA expression is driven by the p43 and the neoantigen peptide uses the AmyQ secretion tag and the R1 ribosome binding site. The repB gene encodes the *Bacillus* replication protein that allows for a high plasmid copy number. The plasmid also contains an ampicillin resistance gene and origin of replication for *E. coli* to allow for plasmid construction in an *E. coli* MC1061 F'.

[0043] Figure 3 is a schematic of representative neoantigen peptide transcriptional units. In the first example, the promoter in front of the neoantigen p53_001 sequence is the p43 (constitutive) promoter, the ribosome-binding site (RBS) is R0 and the terminator is B0015. Theoretically, different combinations of the promoters, RBSs, secretion tags, and mature neoantigen peptide sequences can be used to produce the most efficient construct for a given target organism as seen in the other two constructs.

[0044] Figure 4 is a schematic of plasmid pGP8002 (the sequence of which is set out in SEQ ID NO: 41), used to engineer *E. coli* for the expression of cancer neoantigens. The plasmid

contains the transcriptional units for dapA, adjuvant gene, and the Tp53 neoantigen. In this case, the neoantigen is the p53 sequence from amino acid number 160 to amino acid 185, with a single mutation, R175H. In this case, dapA expression is driven by the J23109 promoter and the neoantigen peptide and adjuvant proteins under the expression of the same J23106 promoter.

[0045] Figure 5 is a schematic of plasmid pGP8003 (the sequence of which is set out in SEQ ID NO: 42), used to engineer *E. coli* for the expression of cancer neoantigens. The plasmid contains the transcriptional units for dapA and an adjuvant - Tp53 neoantigen fusion protein. In this case, the neoantigen is the p53 sequence from amino acid number 165 to amino acid 185, with a single mutation, R175H. In this case, dapA expression is driven by the J23109 promoter and the neoantigen-adjuvant fusion protein is under the expression of the J23106 promoter.

[0046] Figure 6 is a schematic of representative neoantigen peptide transcriptional units. In the first example (SEQ ID NO: 43), the adjuvant and p53_001 neoantigen genes are located on an operon expressed under the same J23106 promoter. In the second example (SEQ ID NO: 44), the neoantigen peptide is fused to the 3' end of the adjuvant gene and expressed under the J23106 promoter as one fusion protein. The ribosome-binding site (RBS) is BCD2 (SEQ ID NO: 45) and the terminator is B0015.

DETAILED DESCRIPTION

[0047] Using a novel approach to strain selection, *Escherichia coli* and *Bacillus* spp. provided herein are selected and genetically engineered to produce and secrete heterologous patient-specific neoantigen peptides. These genetically engineered strains can then be used to induce a targeted immune response against cancer cells anywhere in the patient's body.

[0048] The present disclosure is based, at least in part, on unexpected findings showing that not all bacteria can be engineered to colonize the gut of animals or humans and maintain the metabolic activity for protein production. *E. coli* and *Bacillus* isolates provided herein are thus selected based on localization and metabolic activity in intestinal tract conditions.

[0049] The present disclosure is also based, at least in part, on unexpected findings showing that not all bacteria can be engineered to express and secrete neoantigen peptides because of specific intracellular and extracellular proteases encoded in the genome of the bacteria that enzymatically digest peptides. *E. coli* and *Bacillus* isolates provided herein are thus selected based on the absence of such proteases from their genome.

[0050] The present disclosure is also based, at least in part, on unexpected findings showing that not all secretion systems can be used to secrete specific neoantigen peptides because of unpredictable biophysical interactions between neoantigen peptides and bacterial cell membranes and cell membrane proteins that secrete molecules out of the bacterial cell. *E. coli* and *Bacillus* isolates and secretion tags provided herein are thus selected based on the secretion strength of example neoantigen peptides.

[0051] *E. coli* and *Bacillus* isolates provided herein are selected based on the absence of virulence factors, other pathogenicity factors, and/or antibiotic resistance.

[0052] The *E. coli* and *Bacillus* isolates selected herein are then genetically engineered to express neoantigen peptides.

[0053] Numerous tools and protocols exist for genetic engineering of *E. coli* and *Bacillus*, many of which were originally developed for the model strains, *E. coli* Nissle 1917 [Praveschotinunt, *et al.* Nat Commun 10, 5580 (2019)] and *Bacillus subtilis* 168 [Guiziou *et al.*, Nucleic Acids Research, 44, 7495 (2016); BGSC – Home, bgsc.org].

[0054] Genetic constructs are engineered to contain polycistronic DNA constructs that consist of polynucleotides encoding a bacterial promoter, ribosome binding site, secretion tag, one or more neoantigen peptides, and terminator sequence. The neoantigen peptides are specific to each patient.

[0055] *E. coli* and *Bacillus* isolates are transformed with libraries of the genetic constructs. Libraries of genetic constructs are screened for immunogenic activity in vitro to evaluate peptide production. Genetically engineered *E. coli* and *Bacillus* isolates are tested for in vitro immunogenic activity.

[0056] Genetically engineered *E. coli* and *Bacillus* isolates are tested for in vivo immunogenic activity and for in vivo activity against cancer. The host animal can be of small mammal of the order Rodentia. The host animal can be of the species *Mus musculus* (mouse). The cancers of interest can be solid cancers, e.g., colon cancer, breast cancer, and pancreas cancer, or hematological cancers, e.g., acute lymphoblastic leukemia, acute myeloid leukemia, and chronic lymphocytic leukemia, or pediatric cancers, e.g. leukemia, brain and spinal cord tumors, neuroblastoma, Wilms tumor, and lymphoma.

Selection of Probiotic *E. coli* and *Bacillus* spp.

[0057] *E. coli* and *Bacillus* isolates can be commercially available, including, but not limited to *E. coli* Nissle 1917 (Sonnenborn U., FEMS Microbiology Letters, Volume 363, Issue 19, October 2016, fnw212, doi: 10.1093/femsle/fnw212), Symbioflor 1, Symbioflor 2, *Bacillus subtilis* DSM 29784 and DSM 17299, and *Bacillus Licheniformis* DSM 28710 (Lee *et al.* Food Sci Biotechnol. 2019 Oct; 28(5): 1297–1305. doi: 10.1007/s10068-019-00691-9).

[0058] *E. coli* and *Bacillus* spp. isolates can be proprietary strains including, but not limited to General Probiotics 673 (GP673), GP700, GP1191 (Kaznessis *et al.*, WO 2020/139852 A1 WO 2021/154872 A1), GP1252 and GP1336 (Kaznessis *et al.*, PCT/US23/69266), GP8001, GP8002, GP8003, GP9001, GP9002, and GP9003. *Escherichia coli* strain GP700 has been deposited with the American Type Culture Collection (ATCC) and assigned ATCC Accession No. PTA-126596. *Bacillus paralicheniformis* strain GP1336 has been deposited with the American Type Culture Collection (ATCC) and assigned ATCC Accession No. PTA-127307.

[0059] *E. coli* and *Bacillus* spp. can be isolated from the stool samples of cancer patients as described, e.g., in Example 1.

[0060] Isolated *E. coli* and *Bacillus* spp. colonies can be tested using DNA fingerprinting colony PCR (cPCR) to identify unique isolates, as described for example in Example 2.

[0061] *E. coli* and *Bacillus* spp. can be characterized using 16S ribosomal RNA sequencing, as described, e.g., in Example 3.

[0062] *Bacillus* spp. isolated according to the disclosure include, for example, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus paralicheniformis*, *Bacillus coagulans*, *Bacillus pumilus*, *Bacillus clausii*, or *Bacillus amyloliquefaciens*.

[0063] Isolated *E. coli* can be tested for the presence of virulence and pathogenic factors, including but not limited to, stx1, stx2, estA, estB, LTI, LTII, sigA, sepA, hylEtoC, and ibeA. *E. coli* are selected based on the absence of these factors.

[0064] Isolated *Bacillus* spp. can be tested for the presence of virulence and pathogenic factors, including but not limited to, T7SS, ALO, anthrax toxin, cerulide, certhrax, cytK, HBL, nhe, inhA, capsule, bacillibactin, hal, ilsA, petrobactin, atxA, and bslA. *Bacillus* spp. are selected based on the absence of these factors.

[0065] *E. coli* and *Bacillus* spp. can be tested for neoantigen peptide inactivation (referred to as protease activity), as described, e.g., in Example 4. *E. coli* and *Bacillus* spp. are selected for low protease activity.

[0066] *E. coli* and *Bacillus* spp. are tested for the presence of intracellular, membrane and extracellular proteases, including but not limited to, nprE, nprB, AprE, WprA, Vpr, Bpr, Epr, HtrA, and HtrB. *E. coli* and *Bacillus* spp. are selected for the absence of these proteases.

[0067] *E. coli* and *Bacillus* spp. can be tested for their susceptibility to antibiotics, including but not limited to, kanamycin, chloramphenicol, tetracycline, enrofloxacin, and amoxicillin. *E. coli* and *Bacillus* spp. can be selected for being susceptible to antibiotics as described, e.g., in Example 5.

[0068] *E. coli* and *Bacillus* spp. can be tested for hemolytic activity. *E. coli* and *Bacillus* spp. can be selected for not exhibiting hemolytic activity, as described, e.g., in Example 6.

Construction of Synthetic DNA Sequences

[0069] The method of construction of synthetic DNA sequence involves utilizing standard molecular cloning techniques, such as restriction enzyme-based cloning or Gibson assembly, for engineering the expression of neoantigens in *E. coli*. The neoantigen-encoding genes and any necessary genes encoding adjuvants, obtained from genome sequencing data or gene synthesis, can be ligated into a suitable *E. coli* expression vector, such as pUC19 or pET-28a, at specific restriction enzyme sites or by seamless assembly using overlapping DNA fragments. The recombinant plasmids can then be transformed into *E. coli* competent cells using established transformation protocols. This embodiment enables efficient cloning and expression of neoantigens in *E. coli*, facilitating their production for incorporation into Personalized Bacterial Cancer Vaccines.

[0070] In another example, the method includes employing *Bacillus*-specific cloning techniques for engineering the expression of neoantigens in *Bacillus* spp. *Bacillus*-compatible vectors, such as pHT01 or pUB110, can be used for cloning and expression of neoantigen genes in *Bacillus* species. These vectors typically contain suitable restriction enzyme sites or assembly sites for the insertion of neoantigen genes. The cloning process can involve restriction enzyme digestion and ligation, or seamless assembly methods, to generate recombinant plasmids carrying the desired neoantigen sequences. The resulting plasmids can be introduced

into *Bacillus* competent cells using established transformation protocols. This embodiment enables efficient cloning and expression of neoantigens in *Bacillus*, facilitating their production for use in PBCVs.

[0071] *E. coli* and *Bacillus* spp. can be transformed with a DNA plasmid as described, e.g., in Example 7. See Example 8 for a description of exemplary plasmid designs.

Plasmid Backbone and Architecture

[0072] The plasmids used in *E. coli* and *Bacillus* contain an origin of replication. The plasmid can contain an *E. coli* origin of replication. The plasmid can contain a *Bacillus* origin of replication such as the *Bacillus* origin of replication pUB110 or derivatives thereof.

[0073] The plasmids contain a selectable marker for the modified *E. coli* and *Bacillus* spp. The selectable marker can be a kanamycin or a chloramphenicol resistance gene. The selectable marker can be a functional *dapA* or *thyA* gene isolated from a *E. coli* or *Bacillus* spp. isolate, respectively.

[0074] The plasmids contain a promoter region linked to one or more ribosome binding sites which is/are linked to the secretion tag/neoantigen peptide gene fragment fusion (the coding region) which is linked to the terminator. This polynucleotide comprises the neoantigen peptide transcriptional unit (TU). The TU encompasses both the coding region and the regulatory sequence. The TU can polycistronic and includes the secretion tag/neoantigen peptide along with an additional secretion tag/neoantigen peptides or other genes of interest.

[0075] Exemplary *E. coli*-based plasmid systems are pUC19 or pET-28a, neoantigen-encoding genes can be inserted into these plasmids using standard cloning techniques.

[0076] Exemplary final DNA plasmids are pGP8001 (Figure 2), pGP8002 (Figure 4), pGP8003 (Figure 5), pKG293, pSM504, pSM547-pSM552, pSM576, pSM583, pSM584, pSM587, or pSM595-pSM600 for *Bacillus* spp.

DNA Promoters

[0077] For *E. coli*, the promoter used to express the secretion tag/neoantigen peptide fusion can be, for example, pLacI or variants thereof, PlacZ or variants thereof, GlnRS or variants thereof, and T7 or variants thereof.

[0078] For *Bacillus* spp., the promoter used to express the secretion tag/neoantigen peptide fusion can be, for example, the *Bacillus* p43 constitutive promoter or variants thereof, the *Bacillus* pveg constitutive promoter or variants thereof, the *Bacillus* pgsiB promoter or variants thereof, the *Bacillus* pylB promoter or variants thereof, the *Bacillus* pxylA promoter or variants thereof, or the *Bacillus* plial promoter or variants thereof.

[0079] Naturally occurring bacteria monitor environmental conditions and they respond by modifying the expression pattern of their genes. Transcription of genes is carried out by a single species of RNA polymerase (RNAP). The core enzyme of RNAP executes RNA polymerization reactions, but it cannot recognize a DNA promoter, bind to it and initiate transcription.

[0080] The task of promoter recognition in bacteria is left to one of a few protein subunits called sigma factors. Each sigma factor binds to its cognate promoter and connects with the RNAP core enzyme, forming the fully functioning RNAP holoenzyme. In *E. coli* there are seven known sigma factors and each bind to DNA promoters under different conditions. For example, Sigma 70 binds to its cognate DNA promoters at all times. Sigma 38 binds to its DNA cognate promoters in stationary state.

[0081] Thus, expression of a gene of interest can be controlled by employing promoters that interact with sigma factors that are dominant under the desired expression condition. For example, by employing a promoter capable of binding sigma 38 but not sigma 70, gene expression would be upregulated in stationary phase rather than in exponential phase.

[0082] A list of known sigma factors active in *E. coli* is presented in Table 1A.

[0083] A list of known sigma factors active in vegetative *Bacillus* spp. is presented in Table 1B.

Table 1A

Sigma factor	Gene	Purpose of Regulation
σ^{19}	<i>FecI</i>	Regulates iron transport.
σ^{24}	<i>RpoE</i>	Extracytoplasmic/extreme heat stress.
σ^{28}	<i>RpoF</i>	Flagellar control
σ^{32}	<i>RpoH</i>	Heat shock.
σ^{38}	<i>RpoS</i>	Starvation/stationary phase.
σ^{54}	<i>RpoN</i>	Nitrogen-limitation.

σ^{70} / σ^A	<i>RpoD</i>	"Housekeeping" or primary sigma factor.
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Table 1A. Known Sigma Factors for *E. coli* [Gruber TM, Gross CA (2003) Annual Review of Microbiology. 57: 441–66].

Table 1B

Sigma Factor	Gene	Purpose of Regulation
σ^A	sigA, rpoD	Housekeeping
σ^B	sigB	General stress
σ^H	sigH, spoOH	Postexponential, competence, and early sporulation
σ^D	sigD, flaB	Chemotaxis, autolysin, and flagellar control
σ^L	sigL	Degradative enzyme control

Table 1B: Known sigma factors in *Bacillus* spp.

[0084] Table 2A provides a list of example promoters compatible with various *E. coli*.

[0085] Table 2B provides a list of example promoters compatible with various *Bacillus* spp.

Table 2A

Promoter	Description	Source
Constitutive		
Anderson promoter library	Developed from library screen	1
Synthetic promoters J23100, J23106, J23109	iGEM constitutive promoters, SEQ ID NO: 10, 11, 12	1
P(Bla)	Ampicillin Resistance	1
P(Cat)	Chloramphenicol Resistance	1
P(Kat)	Kanamycin Resistance	1
PlacI	lacI promoter	2
PlacZ	lacZ promoter	1
PlacIQ	mutated lacI promoter	3
LacUV5	high expression lacZ promoter	4

GlnRS	glutaminyl-tRNA synthetase	1
T7	Phage	1
SP6	Phage	1
PN25	Phage	5
Exogenously-Induced		
PBAD	Arabinose-Inducible	1
Plac	Lactose-Inducible	1
PTac	Lactose-Inducible	1
PTet, PTetO, PTetA	Tetracycline-Inducible	1
PTrp	Tryptophan-Inducible	1
PCpxP	Glucose-Inducible	6
Pm or Psal	Salicylate-Inducible	7
pH-Inducible		
PgadA	GadA promoter, SEQ ID NO: 2	8
PgadB	GadB promoter, SEQ ID NO: 3	8
PhdeA	HdeA promoter	8
Osmotic Pressure/Salt-Induced		
PosmBp2	OsmB promoter	9
Pgad	Chloride-Inducible promoter	10
PosmC	OsmC promoter	11
Anaerobically-Induced		
PFnrS	small sRNA (FrnS) promoter, SEQ ID NO: 1	12
PynfEFG	YnfEGF operon promoter	13
pNirB	NirB promoter	14
ydfZ	YdfZ promoter	13
frdABCD	FrdABCD operon promoter	13
Starvation-Induced		
osmBp2	OsmB promoter, SEQ ID NO: 4	9
PmcjA	Microcin J25 mcjA native promoter	15

PmcjB	Microcin J25 mcjB native promoter	15
PmcjC	Microcin J25 mcjC native promoter	15
PyciG	YciG promoter	16
PkatE	KatE promoter	16
PgadA	GadA promoter, SEQ ID NO: 2	16
PosmY	OsmY promoter	16
Temperature-Induced		
pTlpA	Induced at high temperatures	17
pR-pL	Induced at high temperatures	18

Table 2A. Examples of *E. coli* DNA promoters.

Sources for Table 2A: 1. parts.igem.org/Promoters/Catalog/Ecoli/Constitutive; 2. Piraner et al. Nat Chem Biol. 2017 Jan;13(1):75-80; 3. Suhyun K, et al. ACS Synth. Biol. 2018, 7, 9, 2270-2281; 4. Schlegel S, et al. Cell Mol Life Sci. 2017 Mar;74(5):891-908; 5. Kammerer W, et al. EMBO J. 1986 Nov; 5(11): 2995–3000; 6. Courbet A, et al. Sci Transl Med. 2015 May 27;7(289):289ra83; 7. Royo JL, et al. Nat Methods. 2007 Nov;4(11):937-42; 8. Tucker DL, et al., J. Bacteriol. 183: 6551-6558; 9. Boulanger A, et al. J Bacteriol. 2005 May;187(9):3282-6; 10. Sanders JW, et al. Appl Environ Microbiol. 1997 Dec;63(12):4877-82.; 11. Gutierrez C, et al. J Mol Biol. 1991 Aug 20;220(4):959-73.; 12. Boysen A, et al. J Biol Chem. 2010 Apr 2;285(14):10690-702; 13. Kang Y, et al. J Bacteriol. 2005 Feb; 187(3): 1135–1160; 14. Nasr R, et al. Jundishapur J Microbiol. 2014 Jul; 7(7): e15942.; 15. Chiuchiolo MJ, et al., J Bacteriol. 2001 Mar; 183(5): 1755–1764.; 16. Shimada T, et al, J Bacteriol. 2004 Nov;186(21):7112-22; 17. Hurme R, et al Cell. 1997 Jul 11;90(1):55-64.; 18. Valdez-Cruz NA, et al., Microb Cell Fact. 2010 Mar 19; 9:18.;

Table 2B

Promoter	Description	Sequence	Article
	Constitutive		
Pveg	Strong constitutive promoter	SEQ ID NO: 23	Radeck et al., Journal of Biological Engineering, 7, (2013)

p43	Constitutive promoter	SEQ ID NO: 24	Wang and Doi, The Journal of Biological Chemistry, 259, 8619-8625 (1984)
	Stationary Phase-Induced		
pgsiB	Induced by heat, ethanol, acid shock as well as glucose and phosphate starvation	SEQ ID NO: 25	Paccez <i>et al.</i> , Vaccine, 24, 2935- 2943 (2006); Nguyen <i>et al.</i> , Plasmid, 54, 241- 248 (2005)
pylB	Induced by Rhamnose, Repressed by Glucose	SEQ ID NO: 26	Yu <i>et al.</i> , Scientific Reports 5:18405, 1-9 (2015)
	Inducible		
pxyla	Xylose-inducible	SEQ ID NO: 27	Popp <i>et al.</i> , Scientific Reports, 7:15058, 1-13 (2017)
plial	Bacitracin-inducible	SEQ ID NO: 28	Popp <i>et al.</i> , <i>supra</i>

Table 2B: Examples of *Bacillus* spp. promoters

[0086] Promoters for *E. coli* used herein include but are not limited to, high, medium, and low expression constitutive promoters, promoters that respond to stress, nutrient limitations, varying pH, varying osmotic pressure, and promoters that activate in stationary state.

[0087] Constitutive promoters perform best in nutrient-rich environments of the GI tract - their differences in strength of gene expression are also used as a way to produce neoantigen peptides, maturation factors and secretion machinery at the most optimal ratios.

[0088] The FNR promoter acts as a constitutive control in the most anerobic environments of the GI tract, as it originates from a switch system in *E. coli* between aerobic and anaerobic metabolism, the FNR regulon.

[0089] GadA/B promoters are pH sensitive, which makes them useful for the highly acidic components of the GI tract.

[0090] The rpoS promoters can be used. In various embodiments herein, anaerobically-inducible promoters can be used. In various embodiments herein, chloride-inducible promoters can be used. In various embodiments herein, stationary-phase promoters can be used.

[0091] In various embodiments, promoter osmB is a stress-responsive rpoS promoter intended for nutrient-poor environments with a high salt/ion content (osmotic stress).

[0092] The pveg promoter in *B. subtilis* is a super constitutive promoter and is the strongest known promoter in *Bacillus*. It is an σ^A -dependent promoter that seems to be essential for the growth of germinating cells coming out of the sporulation stage. Pveg is a popular promoter to use for heterologous protein production, both plasmid and genome-based, due to its strong activity.

[0093] The p43 promoter is another popular constitutive promoter used for heterologous protein production in *Bacillus*. One reason why p43 is a popular promoter to use is because its region has overlapping binding sites for σ^A and σ^B which make it active during both exponential and stationary phase. Studies of heterologous protein production with p43 have proven that the target protein is produced from the start of exponential phase all the way to late stationary phase.

[0094] Two popular stationary phase-induced promoters to use for heterologous protein production include the pgsiB and pylB. The promoters are desirable for industry use because they are auto-inducible promoters which means an inducer does not need to be added during manufacturing resulting in reduced production costs. PgsiB is regulated by σ^B and is induced by heat shock, salt or ethanol stress, and glucose limitation. A study investigating the activity of pgsiB promoter under different environmental conditions showed that induction increased 7, 8, and 12-fold under acid, heat, and ethanol shock, respectively. It can be activated as early as mid-exponential phase and reach its peak activity at stationary phase. Additionally, pgsiB has low basal activity when it is not induced and could make it an attractive promoter to use to control the expression of a given target protein.

[0095] PylB is another common auto-inducible promoter that is being explored for industry uses. The promoter is most active during mid-exponential phase all the way through stationary phase. The expression level of pylB is directly correlated with the cell density. Overall, pylB has been shown to produce more of a target protein during exponential and stationary phase than the constitutive promoter p43.

[0096] Common inducible promoters in *B. subtilis* that are used in engineering genetic constructs include pxyla and plial. Pxyla is a promoter that is induced by xylose. The promoter is accompanied with a gene called xylR which acts as the repressor on the promoter. In the presence of xylose, the repressor is released and the promoter is activated through derepression. The lial promoter is regulated by the LiaRS two-component system that responds to presence of bacitracin in sub-lethal amounts. It has a very low level of basal activity and is activated in a concentration-dependent manner.

Ribosome Binding Sites

[0097] The ribosome binding site can be, for example, R0 and variants thereof, R1 and variants thereof, R2 and variants thereof, R3 and variants thereof, R4 and variants thereof, R5 and variants thereof, R6 and variants thereof, and/or R7 and variants thereof.

[0098] Gene expression can be controlled both through manipulation of the transcription efficiency as well as manipulation of the translation efficiency. The ribosome binding site (RBS) largely dictates the efficiency with which the transcribed mRNA is translated into a protein. Numerous ribosome binding sites have been identified for *Bacillus subtilis* 168. Sequences and libraries of these RBSs are publicly available.

[0099] Guiziou *et al.*, *supra* developed multiple libraries of ribosomal binding sites and characterized their relative gene expression in *Bacillus subtilis* 168. Table 3 provides a list of exemplary RBSs and their sequences as described by Guiziou *et al.*, *supra*. These RBSs and derivatives thereof can be used in various *Bacillus* spp. provided herein to control translation of genes of interest.

Table 3: Exemplary RBSs used in *Bacillus* spp.

RBS Name	DNA Sequence (5'-3')	SEQ ID
R0	GATTAATAATAAGGAGGACAAAC	29
R1	GCTCTTAAGGAGGATTTTAGA	30
R2	GGTGGAAAGGAGGTGATCGAC	31
R3	GGTGGGAAGGAGGACATTCGAC	32
R4	TGACATGAAAGGAAGTATTTGAAA	33
R5	GGTGGGAAGGAGGTGATCCA	34
R6	GGTGGGAAGGAGGGGGTTTCGAC	35
R7	GGTGGGAAGGAGGAAGTACT	36

Neoantigen Peptides

[0100] The DNA plasmids include a gene encoding for at least one neoantigen protein.

[0101] A nucleotide sequence encoding a neoantigen peptide may be predicted based on reference to the standard genetic code. When a neoantigen peptide is to be expressed in a particular microbe, a nucleotide sequence encoding the neoantigen peptide may be produced with reference to preferred codon usage for the particular microbe.

[0102] Genetically engineered bacteria herein can express and secrete one or more neoantigen peptides.

Secretion Tags

[0103] In one example, the method involves utilizing secretion systems in *E. coli* for efficient secretion of neoantigens. Examples of secretion systems suitable for *E. coli* include:

[0104] 1. Sec System: The Sec secretion system is a widely used pathway in *E. coli* for protein translocation across the cytoplasmic membrane. By incorporating the appropriate signal peptide sequence upstream of the neoantigen gene in the expression plasmid, the neoantigen can be targeted for secretion via the Sec pathway.

[0105] 2. TAT System: The Twin-Arginine Translocation (TAT) secretion system is another secretion pathway in *E. coli* that allows the translocation of folded proteins across the cytoplasmic membrane. By utilizing the TAT system, the neoantigen can be engineered with a TAT signal peptide for efficient secretion.

[0106] In another example, the method includes utilizing secretion systems in *Bacillus* spp. for efficient secretion of neoantigens. Examples of secretion systems suitable for *Bacillus* include:

[0107] 1. Sec System: Similar to *E. coli*, the Sec secretion system is also present in *Bacillus* spp. By incorporating the appropriate signal peptide sequence upstream of the neoantigen gene in the expression plasmid, the neoantigen can be targeted for secretion via the Sec pathway in *Bacillus* strains.

[0108] 2. Tat System: The Tat secretion system, including TatA, TatB, and TatC proteins, is functional in *Bacillus subtilis*. By incorporating a Tat signal peptide, the neoantigen can be directed for secretion through the Tat pathway in *Bacillus* strains.

[0109] The secretion tag sequence is fused to the mature neoantigen peptide sequence. The secretion tag sequence can be derived from *Bacillus* spp. such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus paralicheniformis*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*. The secretion tag can be a sec-dependent or tat-dependent secretion tag. The sec-dependent secretion tag can be the AmyQ secretion tag, the LipA secretion tag, the Mdr secretion tag, the AprE secretion tag, the AbnB secretion tag, the EglS secretion tag, the PhrC secretion tag or the LipA secretion tag. The tat-dependent secretion tag can be the PhoD secretion tag or the YwbN (EfeB) secretion tag. The secretion tag can be a variant of the aforementioned secretion tags.

[0110] A coding sequence encoding a neoantigen peptide may further include nucleotides encoding a secretion signaling protein, such that the neoantigen peptide and the secretion signaling protein are fused and expressed as a single protein. A secretion signaling protein, or

secretion tag, targets a protein for secretion out of the cell, and is usually present at the amino-terminal end of a protein. Secretion signaling proteins useful in prokaryotic microbes are known in the art and routinely used.

[0111] As used herein, 'secretion tags' or 'secretion signaling proteins' are protein sequences that are directly upstream of the mature protein that would be secreted out of the cell. For reviews on secretion and the use of secretion tags for heterologous gene expression in *Bacillus*, refer to Fu *et al.* [Biotechnology Advances, 25, 1–12 (2007)] and Simonen and Palva [Microbiological Reviews, 57, 109–137 (1993)].

[0112] The Sec and Tat secretion pathways are common routes for secretion from *Bacillus* spp. The regions of a secretion tag that uses the Sec pathway include a positively charged NH₂ terminus followed by a hydrophobic region in the middle termed the H region and ends with a polar C region where the cleavage site is located. The consensus cleavage motif for a Sec-dependent secretion tag is typically A-X-A or V-X-A but this is not always the case. The secretion tags can serve many purposes such as preventing premature folding of protein and the degradation of the protein in the cell. Most importantly the secretion tags guide the protein to the correct secretion machinery at the membrane. During or directly after translocation, designated type I signal peptidases (SPase) cut the mature protein away from the secretion tag at the cleavage site. After the successful cleavage of the secretion tag from the mature peptide, the tag is subsequently degraded by SppA and TepA because accumulation of signal peptides can inhibit secretion.

[0113] After the precursor protein is ribosomally-synthesized, a signal recognition particle (SRP) forms a ribonucleoprotein complex with the signal peptide. The SRP consists of a small condition RNA (scRNA) with a Ffh (GTPase) protein and two molecules of the Hbsu protein. After a successful interaction, the SRP guides the complex to a membrane protein called FtsY where precursor protein is then passed along to the Sec translocases. The motor component of the translocation machinery for the Sec-dependent pathway is the SecA protein providing the energy via ATP hydrolysis domain for the translocation. The SecA is a transmembrane protein essential for targeting and translocation the desired secreted protein. The SecY, SecE, SecG, and SecDF proteins are integral proteins involved in the translocation of the unfolded mature target proteins across the membrane. In addition to the translocation, there are also several chaperone proteins located in the cytoplasm and extracellular space that aid in protein folding and ensure there is minimal aggregation of protein in the cell.

[0114] *E. coli* is favorable for engineering PBCVs because of the available tools for heterologous protein production and because of the available tools for manufacturing.

[0115] *Bacillus* spp. are favorable for production of heterologous protein due to absence of an outer membrane which would theoretically simplify translocation and secretion. Additionally, *Bacillus* spp. are known to efficiently secrete a large number of proteins, the majority of which use a general secretion pathway.

[0116] Despite their natural propensity for protein secretion, there are a number of hurdles to overcome to successfully secrete heterologous proteins from *E. coli* and *Bacillus* spp. For example, one of the primary challenges encountered is protease activity. *Bacillus* spp. often secrete large quantities of proteases to break down proteins in the environment. If a target protein is small and linear it can be easily degraded at a higher rate than it can be produced. Other issues that can arise could be interactions with the secretion tag and target proteins and interactions with the secretion machinery.

[0117] Table 4 shows the different exemplary Sec and Tat-dependent secretion tags that were fused to mature peptides as described in the Examples. Included with each secretion tag is the amino acid sequence that ends with the cleavage site where the tag is cleaved from the mature peptide. The cleavage site is generally recognized by the A-X-A or V-X-A motif (*italicized*) though this is not the case for every Sec-dependent secretion tag. Also listed is the function of the protein that the secretion tag is originally attached to in *B. subtilis* 168's genome (exception is AmyQ which is found in *B. amyloliquefaciens*'s genome).

Table 4: Examples of Sec and Tat-dependent secretion tags for *Bacillus* spp.

Secretion Tag	Amino Acid Sequence	Wild-Type Protein Function
AmyQ	MIQKRKRTVSFRLVLMCTLLFVSLPITKTSA (SEQ ID NO: 13)	Amylase (<i>B. amyloliquefaciens</i>)
*LipA	MKFVKRRRIALVTILMLSVTSLFALQPSAKAA (SEQ ID NO: 14)	Alkaliphilic lipase
Mdr	MDTTTAKQASTKFVVLGLLLGLMSAMDNTIVATA (SEQ ID NO: 15)	Multi-drug efflux transporter
AprE	MRSKKLWISLLFALTIFTMAFSNMSAQA (SEQ ID NO: 16)	Serine alkaline protease (Ca ²⁺ -dependent <i>subtilisin</i> E)
PhrC	MKLKSKLFVICLAAAAIFTAAGVSANA (SEQ ID NO: 17)	secreted regulator of the activity of phosphatase RapC and competence and sporulation stimulating factor (CSF)

AbnA	MKKKKTWKRFLHFSSAALAAGLIFTSAAPAEA (SEQ ID NO: 18)	arabinan-endo-1,5-alpha-L-arabinase
AbnB	MFNRLFRVCFLAALIMAFTLPNSVYA (SEQ ID NO: 19)	arabinan-endo-1,5-alpha-L-arabinase (Ca ²⁺ -dependent)
EglS	MKRSISIFITCLLITLLTMGGMIASPASA (SEQ ID NO: 20)	endo-1,4-beta-glucanase
PhoD	MAYDSRFDEWVQKLKEESFQNNTFDRRKFIQGAG KIAGLSLGLTIAQSASA (SEQ ID NO: 21)	alkaline phosphatase D
YwbN (EfeB)	MSDEQKKPEQIHRRDILKWGAMAGAAVAIGASGLG GLAPLVASA (SEQ ID NO: 22)	peroxidase

[0118] The secretion tags derived from *Bacillus subtilis* such as *Bacillus* 168 can be inserted upstream of a neoantigen peptide gene fragment to generate a secretion tag library, e.g., as described in Example 9.

[0119] The secretion tag library with the neoantigen peptide is transformed into a desired *Bacillus* spp. isolate.

Genetic Manipulation of Modified Strains

[0120] *E. coli* and *Bacillus* spp. provided herein are made competent and transformed with the engineered plasmid, e.g., as described in Example 7.

[0121] *E. coli* and *Bacillus* spp. provided can be made rifampicin resistant, e.g., as described in Example 10.

[0122] An essential gene (ex. *dapA* or *thyA*) can be knocked out of the genome of provided *E. coli* or *Bacillus* spp. yielding an auxotroph, e.g., as described in Example 11.

[0123] Exemplary genetically engineered *E. coli* isolates GP_8001, GP_8002, and GP_8003 and *Bacillus* isolates GP_9001, GP_9002, and GP_9003 are provided herein.

In vitro Assays Mimicking the Intestinal Tract

[0124] Survival of modified or unmodified *E. coli* and *Bacillus* spp. can be tested in intestinal tract-mimicking environments (ex. low pH, presence of bile salts); or in intestinal contents (ex. stomach/gizzard contents, duodenum contents, jejunum contents, ileum contents, large intestine contents, ceca contents). Growth of modified or unmodified *E. coli* and *Bacillus* spp. are tested in intestinal tract-mimicking environments (ex. minimal medium, rich medium); or in

intestinal contents (ex. stomach/gizzard contents, duodenum contents, jejunum contents, ileum contents, large intestine contents, ceca contents, mucus from various animal hosts, or combinations of intestinal contents and laboratory-derived medium), e.g., as described in Example 12.

Collection of Normal and Cancer Tissue Samples

[0125] Tissue and cell samples from cancer patients can be obtained through tissue biopsy procedures. Tissue biopsies are widely used and involve the surgical removal of a small portion of the cancerous tissue for diagnostic and research purposes. Common techniques for tissue biopsy include: a) Needle Biopsy: Utilizing fine-needle aspiration or core needle biopsy techniques, a needle is inserted into the tumor site to extract tissue samples. This minimally invasive approach allows for sampling of solid tumors in various locations. b) Endoscopic Biopsy: this method involves performing endoscopic biopsies to collect tissue samples from internal organs or cavities. Endoscopic procedures allow for direct visualization and targeted biopsy sampling. Common examples include: i) Bronchoscopy: In this procedure, a flexible tube with a camera (bronchoscope) is inserted through the nose or mouth to collect tissue samples from the lungs or airways. ii) Colonoscopy: This procedure involves the insertion of a flexible tube with a camera (colonoscope) through the rectum to collect tissue samples from the colon or rectum. c) Surgical Biopsy: In cases where a larger tissue sample is required, open surgical biopsy procedures may be performed. These include excisional biopsy, incisional biopsy, or punch biopsy, involving the removal of the entire tumor or a representative portion of it.

[0126] Blood samples can be obtained from cancer patients. Blood samples provide access to circulating cancer cells, tumor-derived DNA, and other components that can aid in the identification of neoantigens. Common methods for blood sample collection include: a) Venipuncture: This widely used method involves the insertion of a needle into a vein, typically in the arm, to draw blood. The collected blood can be processed to isolate different components, such as white blood cells or cell-free DNA. b) Liquid Biopsy: Liquid biopsies are non-invasive methods that involve the collection of blood or other bodily fluids, such as plasma or serum, to detect tumor-derived genetic material or circulating tumor cells. Techniques like circulating tumor DNA (ctDNA) analysis or circulating tumor cell (CTC) isolation can be employed.

Whole-Genome Sequencing and Identification of DNA Sequence Differences.

[0127] The tissue/cell samples collected from cancer patients can be subjected to whole-genome sequencing (WGS). WGS is a comprehensive approach that involves sequencing the entire genome of cells, providing a detailed view of the patient's genetic makeup. WGS allows for the identification of DNA sequence differences between normal and cancerous tissues, aiding in the detection of genetic alterations and the subsequent identification of neoantigens.

[0128] Examples of WGS technologies include, but are not limited to, A) Illumina sequencing: Illumina sequencing, also known as sequencing by synthesis (SBS), is one of the most widely used WGS technologies. It utilizes reversible terminators and fluorescently labeled nucleotides to sequence DNA. B) Pacific Biosciences (PacBio) sequencing: PacBio sequencing, also known as single-molecule real-time (SMRT) sequencing, is based on the detection of DNA polymerase activity. It involves the real-time observation of DNA synthesis using fluorescently labeled nucleotides. C) Oxford Nanopore sequencing: Oxford Nanopore sequencing is a nanopore-based technology that measures changes in electrical current as DNA strands pass through nanopores. D) Ion Torrent sequencing: Ion Torrent sequencing is based on the detection of hydrogen ions released during DNA synthesis. It utilizes semiconductor technology to measure the ion flux caused by nucleotide incorporation.

[0129] The cancer and normal tissue/cell samples undergo whole-exome sequencing (WES). WES focuses on sequencing the exons, which are the protein-coding regions of the genome. By targeting the exome, which constitutes only a small fraction of the entire genome, WES provides a cost-effective alternative to WGS. It enables the identification of somatic mutations, including single-nucleotide variants (SNVs) and small insertions/deletions (indels), which contribute to the identification of neoantigens.

[0130] Examples of WES technologies include, but are not limited to, A) Agilent SureSelect: Agilent SureSelect employs hybrid capture technology, where biotinylated RNA probes are used to capture and enrich the exonic regions of interest. The captured DNA fragments are then sequenced using next-generation sequencing (NGS) platforms. B) NimbleGen SeqCap EZ: NimbleGen SeqCap EZ uses solution-based hybrid capture technology, where DNA libraries are enriched using biotinylated RNA probes specific to the exome regions. The captured DNA fragments are subsequently sequenced using NGS technologies. C) Illumina TruSeq Exome: Illumina TruSeq Exome utilizes probe-based capture technology to selectively capture and sequence the exonic regions. The captured DNA fragments are then sequenced using Illumina sequencing chemistry. D) Twist Bioscience Exome Panel: Twist Bioscience offers an exome

enrichment panel that utilizes their proprietary target capture technology. The panel consists of custom-designed, single-stranded DNA probes that hybridize to the exonic regions. The captured DNA is then sequenced using NGS platforms.

[0131] The cancer and normal tissue/cell samples can be subjected to targeted panel sequencing. Targeted panel sequencing involves selectively sequencing a panel of genes known to be involved in cancer development and progression. This approach offers high sequencing depth and sensitivity, allowing for the detection of low-frequency mutations. Targeted panel sequencing can be customized to include genes relevant to the specific cancer type, facilitating the identification of neoantigens associated with the patient's cancer.

[0132] The cancer and normal tissue/cell samples can undergo RNA sequencing (RNA-Seq). RNA-Seq involves the sequencing of the transcriptome, providing insights into gene expression patterns and identifying differentially expressed genes between normal and cancerous tissues. RNA-Seq analysis aids in the identification of fusion genes, alternative splicing events, and the quantification of gene expression levels. This information contributes to the identification of neoantigens and their subsequent incorporation into Personalized Bacterial Cancer Vaccines (PBCVs).

[0133] The cancer and normal tissue/cell samples can be subjected to single-cell sequencing techniques. Single-cell sequencing allows for the analysis of individual cells, enabling the characterization of intra-tumoral heterogeneity and the identification of rare cell populations. Methods such as single-cell RNA sequencing (scRNA-Seq) and single-cell DNA sequencing (scDNA-Seq) can provide insights into gene expression profiles, somatic mutations, and clonal evolution within the tumor. These findings can contribute to the identification of patient-specific neoantigens for the development of PBCVs.

[0134] The cancer and normal tissue/cell samples can be subjected to other sequencing techniques, such as methylome sequencing, chromatin immunoprecipitation sequencing (ChIP-Seq), or other emerging technologies. The choice of sequencing method can be based on the specific requirements of the cancer type, the level of genomic information desired, and the available resources.

Identification and Characterization of Patient-Specific Neoantigens

[0135] A method for neoantigen discovery by comparing sequences of cancer and normal cells can involve aligning the sequences obtained from cancer cells and normal cells to a

reference genome, followed by variant calling. This process compares the differences between the two sequences at the nucleotide level, identifying single-nucleotide variants (SNVs), small insertions/deletions (indels), and other genomic alterations. By focusing on the variants unique to the cancer cells, neoantigens can be identified as those derived from mutated genes or gene regions.

[0136] A method for neoantigen discovery by comparing sequences of cancer and normal cells can utilize prediction algorithms to identify neoantigens based on the identified genetic variants. Various computational tools and algorithms are available that predict neoantigens based on factors such as peptide binding affinity to major histocompatibility complex (MHC) molecules and the likelihood of immunogenicity. These algorithms analyze the genetic alterations identified in the cancer cells and predict the potential neoantigens that can elicit an immune response [Brown SD *et al.*, *Oncoimmunology*. 2018 Dec 22;8(3):1556080. doi: 10.1080/2162402X.2018.1556080].

[0137] Methods for neoantigen discovery and selection can utilize machine learning and artificial intelligence algorithms. These methods include the following steps: 1. Training Datasets: Curating large-scale datasets comprising genomic, transcriptomic, and proteomic data from cancer patients, including information on mutations, gene expression profiles, and protein sequences. 2. Machine Learning Models: Training machine learning models, such as deep neural networks, using the curated datasets to identify features and patterns associated with immunogenic neoantigens. The models learn to predict the immunogenicity and antigenicity of neoantigens based on input features. 3. Neoantigen Prioritization: Utilizing the trained machine learning models to prioritize neoantigens from the identified pool based on their predicted immunogenicity, binding affinity to MHC molecules, and potential to elicit a robust immune response. 4. Incorporating Patient-specific Data: Integrating patient-specific data, such as HLA typing and individual immune profiles, into the AI models to further refine the selection process and ensure personalized neoantigen prioritization. 5. Iterative Optimization: Employing iterative optimization processes where the AI models continuously learn and adapt based on experimental and clinical data. Feedback from immune response evaluations and clinical outcomes is used to refine the models' predictions and enhance the selection of neoantigens.

[0138] Methods for neoantigen discovery and selection can include deep learning algorithms, as described in Cai *et al.*, *Front Oncol*. 2023 Jan 9;12:1054231. doi: 10.3389/fonc.2022.1054231 and references therein. These algorithms include the following steps: 1. Deep Learning Architectures: Designing and training deep neural network

architectures, such as convolutional neural networks (CNNs) or recurrent neural networks (RNNs), specifically tailored for neoantigen prediction. These models learn complex patterns and relationships between genetic variations, gene expression data, and immunogenicity. 2. Integration of Multiple Data Sources: Incorporating diverse data sources, including genomic sequencing data, transcriptomic profiles, and proteomic data, into the deep learning models. This integration allows the models to capture comprehensive information and make accurate predictions regarding the immunogenic potential of neoantigens. 3. Feature Extraction: Utilizing the deep learning models to extract relevant features and representations from the input data that are indicative of neoantigen immunogenicity. This can include identifying mutation patterns, splicing events, gene expression levels, and protein structural characteristics. 4. Predictive Scoring: Assigning predictive scores to neoantigens based on the output of the deep learning models, reflecting their likelihood of eliciting an immune response. The scoring can be used for ranking and selecting the most promising neoantigens for inclusion in PBCVs.

[0139] These example embodiments highlight the utilization of AI-based methods, including machine learning and deep learning, for the optimization of neoantigen selection. By training models on large-scale datasets, incorporating patient-specific information, and employing iterative optimization processes, this disclosure enables the precise and personalized selection of immunogenic neoantigens, enhancing the effectiveness of Personalized Bacterial Cancer Vaccines in treating and preventing cancers.

[0140] Methods for neoantigen discovery and selection can involve isolating and sequencing peptides from cancer cells and normal cells using mass spectrometry techniques. This approach allows for the direct identification of peptides derived from proteins present in the cancer cells. By comparing the peptide sequences from cancer and normal cells, neoantigens can be identified as those that are specific to the cancer cells and absent in the normal cells.

[0141] Methods for neoantigen selection can incorporate immunogenicity assays to evaluate the potential of identified neoantigens to elicit an immune response. These assays can include in vitro tests, such as T-cell activation assays, to assess the recognition and activation of T cells specific to neoantigens. For example, a plethora of immunogenicity assays can be used for specific gene mutations, as described for mutations of Tp53 in Malekzadeh, *et al.*, J Clin Invest. 2019;129(3):e123791. doi.org/10.1172/JCI123791 and references therein. Additionally, animal models or ex vivo assays can be utilized to evaluate the immunogenicity and therapeutic efficacy of the neoantigens in inducing an immune response against cancer cells.

[0142] Methods for neoantigen selection can utilize computational algorithms and bioinformatics tools to predict neoantigens based on the genomic sequences of cancer and normal tissue samples. By applying algorithms that analyze somatic mutations, gene expression data, and binding affinity prediction models, potential neoantigens can be identified. Other models take into account various factors, including antigen processing and presentation, T-cell receptor recognition, and immune system activation. By assessing the immunogenic potential of neoantigens, the computational approach facilitates the ranking and prioritization of candidate neoantigens for incorporation into PBCVs to stimulate an immune response against cancer cells.

[0143] Methods for neoantigen selection can include employing computational tools to predict epitopes derived from cancer-associated mutations and analyzing their binding affinity to human leukocyte antigen (HLA) molecules. By considering the HLA type of the patient, algorithms can identify neoantigens with high affinity for patient-specific HLA alleles. This embodiment enables the selection of neoantigens that have a higher likelihood of generating an effective immune response when included in PBCVs.

[0144] Methods for neoantigen selection can involve integrating transcriptomic data with genomic information. By comparing gene expression profiles between cancer and normal tissues, computational analysis can identify genes that are overexpressed or aberrantly expressed in cancer cells. The identified genes can serve as targets for neoantigen discovery, as their differential expression indicates the potential presence of cancer-specific antigens.

[0145] The described examples illustrate a range of methods for neoantigen discovery by comparing sequences of cancer and normal cells. It should be noted that these examples are not exhaustive, and other approaches, such as epitope prediction algorithms, functional assays, or combination strategies, can also be employed within the scope of the patent. The choice of methods can be based on factors such as the available resources, the desired sensitivity and specificity, and the specific characteristics of the cancer type being targeted.

[0146] Methods for neoantigen selection can focus on genes that are commonly mutated in various types of cancers. Examples include but are not limited to: 1. ****TP53****: TP53 is a tumor suppressor gene that regulates cell division and prevents the formation of tumors. Mutations in TP53 are one of the most frequent alterations found in multiple cancer types, including lung, breast, colorectal, ovarian, and pancreatic cancers. 2. ****KRAS****: KRAS is an oncogene that plays a critical role in cell signaling pathways regulating cell growth and division. Mutations in the KRAS gene are commonly found in several cancers, including colorectal, lung, and

pancreatic cancers. 3. **EGFR**: Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase involved in cell growth and proliferation. Mutations in EGFR are frequently observed in lung adenocarcinoma and other types of lung cancer. 4. **BRAF**: BRAF is a gene that encodes a protein involved in the MAPK signaling pathway, regulating cell growth and division. Mutations in BRAF are prevalent in melanoma, colorectal cancer, and certain types of thyroid cancer. 5. **HER2**: Human Epidermal Growth Factor Receptor 2 (HER2) is a gene that encodes a protein involved in cell growth and division. HER2 amplification or overexpression is often seen in breast and gastric cancers. 6. **BRCA1 and BRCA2**: BRCA1 and BRCA2 are tumor suppressor genes that play a crucial role in DNA repair. Mutations in these genes increase the risk of breast and ovarian cancers, as well as other types of cancer. 7. **PTEN**: PTEN is a tumor suppressor gene that regulates cell growth and division by inhibiting cell survival pathways. Mutations in PTEN are commonly found in several cancers, including breast, prostate, and endometrial cancers. 8. **ALK**: Anaplastic Lymphoma Kinase (ALK) is a gene that encodes a protein involved in cell signaling. ALK rearrangements or fusions are frequently seen in specific types of lung cancer, such as non-small cell lung cancer (NSCLC). 9. **RET**: RET is a gene that encodes a receptor tyrosine kinase involved in cell growth and division. RET mutations and fusions are observed in multiple cancers, including thyroid cancer and certain types of lung cancer. 10. **CDKN2A**: CDKN2A is a tumor suppressor gene that regulates the cell cycle. Mutations in CDKN2A are commonly associated with melanoma and pancreatic cancer.

[0147] Methods for neoantigen selection can focus on inherited genes that are associated with an increased risk of developing certain types of cancer. Examples include but are not limited to:

[0148] 1: BRCA1 and BRCA2 Mutation Detection. This method involves detecting mutations in the BRCA1 and BRCA2 genes, which are associated with an increased risk of breast, ovarian, and other cancers. By analyzing the genomic sequences of BRCA1 and BRCA2 obtained from patient samples, the presence of pathogenic mutations can be identified. These inherited mutations can be utilized for neoantigen selection in the development of Personalized Bacterial Cancer Vaccines (PBCVs) tailored to patients carrying these genetic alterations.

[0149] 2: Lynch Syndrome Gene Detection. This method focuses on detecting mutations in genes associated with Lynch syndrome, such as MLH1, MSH2, MSH6, and PMS2. Lynch syndrome increases the risk of colorectal, endometrial, and other cancers. Through genomic analysis of these genes, the presence of pathogenic mutations can be determined, providing a

basis for selecting neoantigens for incorporation into PBCVs to target the specific cancer associated with Lynch syndrome.

[0150] 3: APC Gene Mutation Detection. This method includes identifying mutations in the APC gene, commonly associated with familial adenomatous polyposis (FAP) and an increased risk of colorectal cancer. By analyzing the genomic sequences of APC, mutations can be identified, and corresponding neoantigens can be selected for inclusion in PBCVs. These personalized vaccines target the specific cancer risk associated with APC mutations in individuals with FAP.

[0151] 4: PTEN Mutation Detection. This method involves detecting mutations in the PTEN gene, associated with Cowden syndrome and an increased risk of breast, thyroid, and other cancers. Genomic analysis of PTEN can reveal pathogenic mutations, enabling the selection of neoantigens for incorporation into PBCVs. These personalized vaccines target the specific cancer risk associated with PTEN mutations in individuals with Cowden syndrome.

[0152] 5: TP53 Germline Mutation Detection. This method focuses on identifying germline mutations in the TP53 gene, associated with Li-Fraumeni syndrome and an increased risk of multiple cancer types. By examining the genomic sequences of TP53, germline mutations can be detected, serving as sources of neoantigens for PBCV development. These personalized vaccines target the specific cancer risks associated with TP53 germline mutations in individuals with Li-Fraumeni syndrome.

[0153] It is important to note that these examples represent a subset of inherited cancer susceptibility genes, and other relevant genes associated with increased cancer risk can also be considered within the scope of the patent. The selection of genes to target can depend on factors such as the prevalence of mutations, the specific hereditary cancer syndrome, and the available genetic information for the patient population.

Validation of Recombinant Microbe for Expression and Secretion of Neoantigens.

[0154] Methods for growing engineered PBCV and testing for the expression and secretion of neoantigen peptides can involve species-specific procedures.

[0155] Example methods for growing *E. coli* can involve the following steps: 1. Start by preparing a suitable culture medium, such as Luria-Bertani (LB) broth or agar, supplemented with appropriate antibiotics if necessary. 2. Inoculate a small volume of the medium with the engineered *E. coli* strain containing the plasmid carrying the neoantigen and any adjuvant

genes. 3. Incubate the culture at the optimal temperature and conditions for *E. coli* growth, typically 37°C with shaking. 4. Monitor the growth of the culture by measuring optical density (OD) at a specific wavelength or by plating serial dilutions on agar plates and counting colony-forming units (CFUs). 5. At a desired time point, harvest a portion of the culture to assess neoantigen expression and secretion.

[0156] Example methods for testing expression and secretion of neoantigens in *E. coli* can include the following examples: 1. Perform cell lysis: Collect a fraction of the bacterial culture and perform cell lysis using appropriate methods such as sonication or freeze-thaw cycles to release cellular contents. 2. Analyze protein expression: Use techniques like SDS-PAGE or Western blotting to detect and analyze the expression of neoantigens in the lysate. This can involve probing with specific antibodies or antigen-specific probes. 3. Assess secretion: Collect the remaining culture supernatant and concentrate it using methods like ultrafiltration or precipitation. Analyze the concentrated supernatant using SDS-PAGE, Western blotting, or other immunological assays to detect and assess the presence of secreted neoantigens.

[0157] Example methods for growing *Bacillus* spp. can involve the following steps: 1. Prepare a suitable culture medium specific to the *Bacillus* species being used, such as nutrient agar or broth supplemented with appropriate antibiotics if required. 2. Inoculate a small volume of the medium with the engineered *Bacillus* strain harboring the plasmid carrying the neoantigen gene. 3. Incubate the culture at the optimal temperature and conditions for *Bacillus* growth, typically 30-37°C. 4. Monitor the growth of the culture by measuring optical density (OD) or by plating serial dilutions on agar plates and counting colony-forming units (CFUs). 5. Harvest a portion of the culture at the desired time point for further analysis of neoantigen expression and secretion.

[0158] Example methods for testing expression and secretion of neoantigens in *Bacillus* spp. can include: 1. Perform cell lysis: Collect a fraction of the bacterial culture and perform cell lysis using methods suitable for *Bacillus* spp., such as enzymatic lysis or sonication. 2. Analyze protein expression: Employ techniques like SDS-PAGE, Western blotting, or mass spectrometry to detect and analyze the expression of neoantigens in the lysate. Use specific antibodies or antigen-specific probes for detection. 3. Assess secretion: Collect the remaining culture supernatant and concentrate it using techniques like ultrafiltration or precipitation. Analyze the concentrated supernatant for the presence of secreted neoantigens using SDS-PAGE, Western blotting, or immunological assays.

[0159] These example embodiments describe the specific steps and procedures involved in growing each bacterial species and testing the robust expression and secretion of neoantigen

peptides. By following these example, one can effectively evaluate the expression and secretion capabilities of the engineered bacteria, ensuring the suitability of the selected bacterial species for the development of PBCVs.

Immunogenicity Assessment of Neoantigens Expressed and Secreted by PBCVs.

[0160] A method for evaluating the immunogenicity of expressed/secreted neoantigens using tumor cell lines, can comprise a. Cultivating tumor cell lines representative of the cancer type being targeted by the PBCV. b. Treating the tumor cell lines with the expressed/secreted neoantigens derived from the engineered bacteria. c. Assessing the immune response by measuring the activation and proliferation of immune cells, such as T cells, natural killer (NK) cells, or antigen-presenting cells (APCs). d. Analyzing the production of immune-related molecules, including cytokines, chemokines, or cytotoxic effector molecules. e. Monitoring the specific recognition and killing of tumor cells expressing the neoantigens by immune cells using techniques such as flow cytometry, cytotoxicity assays, or ELISPOT assays.

[0161] A method for examining the immunogenicity of expressed/secreted neoantigens using dendritic cells (DCs), comprising: a. Cultivating DCs from peripheral blood monocytes or other suitable sources. b. Co-culturing the DCs with the expressed/secreted neoantigens derived from the engineered bacteria. c. Evaluating the maturation and activation of DCs by assessing surface marker expression, cytokine production, or antigen presentation capability. d. Assessing the ability of DCs to prime and stimulate T cells by measuring T cell activation, proliferation, or cytokine secretion. e. Monitoring the interaction between DCs and T cells, such as antigen-specific T cell recognition or antigen-specific T cell receptor (TCR) signaling.

[0162] A method for evaluating the immunogenicity of expressed/secreted neoantigens using lymphocyte populations, comprising: a. Isolating lymphocytes, including peripheral blood mononuclear cells (PBMCs), from blood samples of cancer patients or healthy donors. b. Stimulating the lymphocytes with the expressed/secreted neoantigens derived from the engineered bacteria. c. Assessing lymphocyte activation, proliferation, or cytokine production as indicators of immune response. d. Analyzing the differentiation and expansion of antigen-specific T cell populations using techniques such as flow cytometry, ELISPOT assays, or TCR sequencing. e. Evaluating the cytotoxic activity of lymphocytes against target cells expressing the neoantigens through cytotoxicity assays, such as the chromium release assay or granzyme/perforin assays.

[0163] These examples embodiments describe the use of in vitro tests with various cell lines, including tumor cell lines, dendritic cells, and lymphocyte populations, to assess the immunogenicity of expressed/secreted neoantigens. By conducting these tests, the efficacy of the Personalized Bacterial Cancer Vaccines (PBCVs) in activating and eliciting an immune response against cancer cells expressing the neoantigens can be evaluated.

[0164] A method for evaluating the expression of Interferon-gamma (IFN- γ) in response to expressed/secreted neoantigens, comprising a. Stimulating immune cells, such as T cells or lymphocytes, with the expressed/secreted neoantigens derived from the engineered bacteria. b. Incubating the stimulated cells for a designated period. c. Harvesting the cell culture supernatant. d. Quantifying the expression of IFN- γ using techniques such as enzyme-linked immunosorbent assay (ELISA) or flow cytometry. e. Analyzing the levels of IFN- γ production as an indicator of immune activation and the potential immunogenicity of neoantigens when expressed and selected from PBCVs.

[0165] A method for assessing the production of Tumor Necrosis Factor-alpha (TNF- α) in response to expressed/secreted neoantigens, comprising a. Treating immune cells, such as dendritic cells (DCs) or macrophages, with the expressed/secreted neoantigens derived from the engineered bacteria. b. Incubating the treated cells for a specified duration. c. Collecting the cell culture supernatant. d. Quantifying the production of TNF- α using techniques such as ELISA or multiplex cytokine assays. e. Analyzing the levels of TNF- α production as an indicator of immune activation and the potential immunogenicity of the selected neoantigens when expressed and secreted from PBCVs.

[0166] A method for analyzing the secretion of Interleukin-2 (IL-2) in response to expressed/secreted neoantigens, comprising a. Co-culturing immune cells, such as T cells or lymphocytes, with the expressed/secreted neoantigens derived from the engineered bacteria. b. Allowing the co-cultured cells to incubate for a designated time period. c. Collecting the cell culture supernatant. d. Detecting the secretion of IL-2 using techniques like ELISA or cytokine bead arrays. e. Assessing the levels of IL-2 secretion as an indication of T cell activation and the immunogenic potential of the neoantigens when expressed and selected from PBCVs.

[0167] These example embodiments describe specific genes, such as Interferon-gamma (IFN- γ), Tumor Necrosis Factor-alpha (TNF- α), and Interleukin-2 (IL-2), and the experimental procedures, such as ELISA or cytokine quantification, to evaluate their expression or production in response to the expressed/secreted neoantigens. By measuring the levels of these cytokines, the immune activation and potential immunogenicity of the neoantigens can be assessed,

providing valuable insights into the immune response generated by the Personalized Bacterial Cancer Vaccines (PBCVs).

[0168] A method for evaluating the efficacy of Personalized Bacterial Cancer Vaccines (PBCVs) using mouse xenograft models, comprising a. Establishing tumor xenografts in immunocompromised mice by injecting human cancer cells. b. Administering the recombinant PBCVs orally to the mice. c. Monitoring tumor growth over time through caliper measurements or imaging techniques. d. Assessing the impact of the PBCVs on tumor growth inhibition or regression compared to control groups. e. Analyzing the immune response by examining infiltrating immune cells within the tumor microenvironment using techniques such as immunohistochemistry or flow cytometry. f. Measuring the systemic immune response by evaluating immune cell subsets and cytokine production in peripheral blood or lymphoid organs. g. Evaluating the overall survival and long-term effects of the PBCVs on tumor burden and metastasis.

[0169] A method for evaluating the immunogenicity of Personalized Bacterial Cancer Vaccines (PBCVs) using syngeneic animal models, comprising a. Establishing syngeneic tumor models in immunocompetent mice by implanting murine cancer cells. b. Administering the recombinant PBCVs orally or via other suitable routes to the mice. c. Monitoring tumor growth kinetics using caliper measurements or imaging techniques. d. Assessing the immune response by analyzing immune cell infiltration within the tumor, cytokine production, and antibody responses using techniques such as flow cytometry, ELISA, or immunohistochemistry. e. Evaluating the activation and expansion of tumor-specific T cells using techniques like tetramer staining or TCR sequencing. f. Assessing the impact of the PBCVs on tumor regression, overall survival, and the establishment of immune memory.

[0170] A method for evaluating the systemic immune response induced by Personalized Bacterial Cancer Vaccines (PBCVs) using animal models, comprising a. Administering the recombinant PBCVs orally or via other suitable routes to immunocompetent animals. b. Assessing peripheral immune cell populations, including T cells, B cells, natural killer (NK) cells, and antigen-presenting cells (APCs), by flow cytometry or other suitable techniques. c. Analyzing the production of immune-related molecules, such as cytokines, chemokines, or immunoglobulins, in serum or other biological fluids using techniques like ELISA or multiplex assays. d. Monitoring the activation and functional properties of immune cells, such as cytokine secretion, cytotoxicity, or phagocytosis, using functional assays. e. Assessing the generation of antigen-specific immune responses by evaluating antigen-specific T cell responses, antibody

titers, or memory immune cell populations. f. Analyzing the long-term effects on immune surveillance, tumor control, and prevention of tumor recurrence.

[0171] These example embodiments describe the use of animal experiments, including mouse xenograft models, syngeneic tumor models, and assessments of the systemic immune response, to evaluate the efficacy, immunogenicity, and long-term effects of Personalized Bacterial Cancer Vaccines (PBCVs). These animal models provide valuable insights into the potential therapeutic benefits, immune response, and safety profile.

Manufacturing of Final Formulation of Recombinant PBCVs.

[0172] A method for GMP manufacturing of Personalized Bacterial Cancer Vaccines (PBCVs) in the form of pills for oral delivery, can comprise the following steps:

Step 1: Master Cell Bank (MCB) and Working Cell Bank (WCB) Generation

- a. Generating a Master Cell Bank (MCB) from the engineered bacterial strain expressing the neoantigens, following established GMP guidelines and documentation.
- b. Establishing a Working Cell Bank (WCB) from the MCB to ensure a consistent and renewable source of the engineered bacteria for production.

Step 2: Fermentation and Culture

- a. Scaling up the production of the engineered bacteria in a controlled fermentation system using suitable growth media and conditions.
- b. Monitoring and controlling critical parameters such as temperature, pH, dissolved oxygen, and agitation to achieve optimal growth and productivity.
- c. Harvesting the bacterial culture at the desired growth phase.

Step 3: Purification and Concentration

- a. Employing purification techniques, such as filtration, centrifugation, or chromatography, to separate and purify the bacterial cells from the fermentation broth.
- b. Concentrating the bacterial cells to obtain a high-density bacterial suspension.

Step 4: Formulation and Encapsulation

- a. Formulating the concentrated bacterial suspension with suitable excipients, stabilizers, and additives to ensure stability and oral delivery compatibility.
- b. Encapsulating the formulated PBCVs into pills using pharmaceutical-grade encapsulation equipment, ensuring uniformity and consistent dosing.

Step 5: Quality Assurance/Quality Control (QA/QC) Documentation

- a. Implementing comprehensive QA/QC measures throughout the manufacturing process to ensure product quality, consistency, and compliance with GMP regulations.
- b. Performing rigorous testing of raw materials, in-process samples, and final PBCV products for identity, purity, potency, sterility, endotoxin levels, and other relevant specifications.
- c. Documenting and maintaining detailed records of manufacturing procedures, batch records, testing results, and any deviations or corrective actions taken.
- d. Conducting stability studies to assess the shelf life and storage conditions of the PBCV pills.
- e. Complying with regulatory requirements and submitting appropriate documentation for regulatory approval and compliance.

[0173] This example embodiment describes the GMP manufacturing process of Personalized Bacterial Cancer Vaccines (PBCVs) in the form of pills for oral delivery. The process involves generating cell banks, fermentation and culture, purification and concentration, formulation and encapsulation, and comprehensive QA/QC documentation. Adhering to GMP guidelines and implementing stringent quality control measures ensures the production of high-quality, safe, and effective PBCV pills for oral administration to patients.

Doses and Duration of Oral Administration of the Personalized Bacterial Cancer Vaccine.

[0174] Embodiment: Oral Administration with 0.01 Billion, 0.1 Billion, 1 Billion, 2 Billion, 3 Billion, 4 Billion, 5 Billion, 6 Billion, 7 Billion, 8 Billion, 9 Billions or 10 Billion colony forming units (CFUs) of PBCV once, twice or thrice per day, for one, two, three, four, five, six or seven days a week, for one, two, three, four, five, six, seven, eight or more weeks. The PBCV pills containing the engineered bacteria are administered orally to the patient at a prescribed dose. This embodiment aims to provide an appropriate dose and treatment course intended to enhance the exposure of the immune system to the neoantigens and potentially improve the immune response against the cancer cells.

[0175] Embodiment: Rectal Administration with 0.01 Billion, 0.1 Billion, 1 Billion, 2 Billion, 3 Billion, 4 Billion, 5 Billion, 6 Billion, 7 Billion, 8 Billion, 9 Billions or 10 Billion colony forming units (CFUs) of PBCV once, twice or thrice per day, for one, two, three, four, five, six or seven days a week, for one, two, three, four, five, six, seven, eight or more weeks. The patient is instructed to insert PBCV suppositories into the rectum, allowing for localized delivery of the vaccine to the lower gastrointestinal tract. The suppositories contain the engineered bacteria expressing the neoantigens. The dosage and composition of the aims to target the immune-rich environment of

the lower gastrointestinal tract, allowing direct interaction between the neoantigens and the immune cells present in the local tissue. This localized delivery may enhance the immune response against the cancer cells expressing the neoantigens while minimizing systemic exposure and potential side effects.

[0176] It's important to note that the specific dosage, formulation, and treatment duration for oral or rectal administration may vary based on individual patient characteristics, cancer type, and clinical considerations. Healthcare professionals should determine the optimal anal dosing regimen in consultation with the patient, considering factors such as patient comfort, tolerability, and potential efficacy.

Validation of the Presence of Personalized Bacterial Cancer Vaccines (PBCVs) in the Gut of a Patient and to Assess the Patient's Immune Responses.

[0177] 1. Embodiment: Fecal Analysis for PBCV Detection. In this embodiment, stool samples from the patient are collected and analyzed to validate the presence of PBCVs in the gut. The analysis may involve techniques such as quantitative polymerase chain reaction (qPCR) or DNA sequencing to detect and quantify the specific genetic markers or sequences unique to the engineered bacteria used in the PBCVs. Positive results indicate the colonization and persistence of PBCVs in the gastrointestinal tract.

[0178] 2. Embodiment: Serological Assays for Immune Response Assessment. This embodiment involves conducting serological assays on blood samples from the patient to assess the immune response triggered by the PBCVs. The serological assays may include enzyme-linked immunosorbent assays (ELISAs) or multiplex immunoassays to measure the levels of specific antibodies targeting the neoantigens expressed by the PBCVs. An increase in neoantigen-specific antibody titers over time indicates an immune response against the cancer cells expressing the neoantigens.

[0179] 3. Embodiment: Flow Cytometry Analysis of Peripheral Blood Mononuclear Cells (PBMCs). In this embodiment, peripheral blood samples are collected, and the immune cells, particularly peripheral blood mononuclear cells (PBMCs), are isolated. Flow cytometry analysis is then performed to assess the activation and phenotype of immune cells, such as T cells and natural killer (NK) cells, in response to the PBCVs. Markers of immune cell activation, cytokine production, and expression of immune checkpoint receptors can be measured, indicating an ongoing immune response against the cancer cells expressing the neoantigens.

[0180] These illustrative embodiments describe tests to validate the presence of PBCVs in the gut through fecal analysis and to assess immune responses through serological assays and flow cytometry analysis of blood samples. These tests provide valuable insights into the colonization of PBCVs in the gastrointestinal tract and the activation of the immune system in response to the neoantigens expressed by the PBCVs. By monitoring these parameters, healthcare professionals can evaluate the effectiveness and immunogenicity of the PBCV therapy.

Tests for detecting and measuring cancer antigens associated with specific types of cancer.

[0181] 1. Embodiment: Prostate-Specific Antigen (PSA) Test for Prostate Cancer. The PSA test is a widely used blood test to measure the levels of prostate-specific antigen, a protein produced by the prostate gland. Elevated PSA levels can indicate the presence of prostate cancer or other prostate-related conditions. This embodiment involves performing the PSA test to assess prostate cancer antigen levels in the patient's blood, providing valuable information for prostate cancer diagnosis and monitoring.

[0182] 2. Embodiment: CA-125 Test for Ovarian Cancer. The CA-125 test measures the levels of cancer antigen 125 in the blood. CA-125 is a protein that is often elevated in ovarian cancer. This embodiment involves conducting the CA-125 test to assess the levels of CA-125 in the patient's blood, aiding in the diagnosis and monitoring of ovarian cancer.

[0183] 3. Embodiment: HER2/neu Testing for Breast Cancer. HER2/neu testing is performed to determine the overexpression or amplification of the HER2/neu gene in breast cancer cells. This testing helps identify breast cancer patients who may benefit from targeted therapies that specifically target the HER2 protein. The embodiment involves performing HER2/neu testing on tumor samples obtained from the patient to determine the HER2/neu status and guide treatment decisions.

[0184] 4. Embodiment: Alpha-Fetoprotein (AFP) Test for Liver Cancer. The AFP test measures the levels of alpha-fetoprotein in the blood, which can be elevated in patients with liver cancer or other liver-related conditions. This embodiment includes conducting the AFP test to assess AFP levels in the patient's blood, aiding in the diagnosis and monitoring of liver cancer.

[0185] 5. Embodiment: Carcinoembryonic Antigen (CEA) Test for Colon Cancer. The CEA test is commonly used for the detection and monitoring of colon cancer. CEA is a glycoprotein that is often elevated in individuals with colon cancer. This embodiment involves performing the CEA test to measure the levels of CEA in the patient's blood. Elevated CEA levels can indicate the presence of colon cancer and help in monitoring treatment response and disease progression.

[0186] 6. Embodiment: CD20 Testing for B-cell Lymphoma. CD20 is a cell surface marker expressed on B-cells, and it is often associated with B-cell lymphomas such as non-Hodgkin lymphoma. This embodiment includes conducting CD20 testing on tumor samples or peripheral blood to determine the presence and levels of CD20-expressing cells. CD20 testing helps in the diagnosis, classification, and monitoring of B-cell lymphomas and aids in the selection of appropriate targeted therapies.

[0187] 7. Embodiment: Philadelphia Chromosome Testing for Chronic Myeloid Leukemia (CML). The Philadelphia chromosome is a genetic abnormality commonly found in patients with chronic myeloid leukemia (CML). This embodiment involves performing Philadelphia chromosome testing, such as fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR), on blood or bone marrow samples to detect the presence of the Philadelphia chromosome. This testing is crucial for confirming the diagnosis of CML and guiding treatment decisions, including the use of targeted therapies. 8. Embodiment: Genetic Testing for Pediatric Cancers. Genetic testing plays a significant role in the diagnosis and management of pediatric cancers. This embodiment encompasses various genetic tests, such as chromosomal analysis, next-generation sequencing, or specific gene mutation testing, performed on tumor samples or blood samples from pediatric cancer patients. These tests help identify genetic abnormalities, gene mutations, or inherited cancer predisposition syndromes, enabling personalized treatment approaches and genetic counseling for the patient and their family.

[0188] These illustrative embodiments demonstrate specific tests targeting cancer antigens associated with colon cancer, blood cancers (such as B-cell lymphomas), and pediatric cancers. By employing these tests, healthcare professionals can enhance the accuracy of cancer diagnosis, prognosis, and treatment selection for patients in these specific contexts. It's important to note that advancements in cancer research and diagnostic techniques may introduce new biomarkers or tests specific to these cancer types in the future.

Applications and Benefits of PBCVs. Treatment, Control, and Prevention of Various Cancers.

[0189] Breast Cancer. In one embodiment, the PBCVs are designed for the treatment, control, and prevention of breast cancer. Breast cancer is a prevalent malignancy, and the PBCVs can be tailored to target patient-specific neoantigens derived from genetic alterations observed in breast cancer cells. The vaccines can be administered orally to target the gastrointestinal tract, where a significant portion of the immune system resides, thereby eliciting a specific immune response against breast cancer cells expressing neoantigens.

[0190] Lung Cancer. In another embodiment, the PBCVs are formulated to combat lung cancer. Lung cancer poses significant challenges due to its diverse subtypes and genetic heterogeneity. The PBCVs can be personalized to incorporate neoantigens specific to lung cancer patients, enabling the immune system to recognize and destroy cancer cells in the lungs. Oral administration of PBCVs allows for targeted delivery to the gastrointestinal tract, augmenting the immune response and enhancing therapeutic outcomes.

[0191] Colorectal Cancer. In yet another embodiment, the PBCVs are developed for the treatment, control, and prevention of colorectal cancer. Colorectal cancer is a common malignancy, and PBCVs can be designed to target patient-specific neoantigens found in colorectal cancer cells. By colonizing the gastrointestinal tract and releasing neoantigens, the PBCVs enhance immune recognition and subsequent immune-mediated elimination of colorectal cancer cells, both locally and systemically.

[0192] Prostate Cancer. In another embodiment, the PBCVs are engineered to address prostate cancer. Prostate cancer is a significant health concern, and PBCVs can be personalized to incorporate patient-specific neoantigens derived from genetic alterations observed in prostate cancer cells. The oral administration of PBCVs allows for targeted delivery to the gastrointestinal tract, where a substantial portion of the immune system resides, thereby triggering a specific immune response against prostate cancer cells expressing neoantigens.

[0193] In a further embodiment, the PBCVs are developed to target pediatric cancers. Pediatric cancers present unique challenges, and PBCVs offer a personalized approach to address these malignancies. By incorporating patient-specific neoantigens, the PBCVs can trigger a robust and specific immune response against pediatric cancer cells. The oral administration of PBCVs allows for targeted delivery to the gastrointestinal tract, enhancing the efficacy of the immune response in pediatric cancer patients.

[0194] These illustrative embodiments illustrate a range of cancers for which the Personalized Bacterial Cancer Vaccines (PBCVs) can be employed. It should be understood that the examples provided are not exhaustive, and the PBCVs can be customized to target various other cancer types, including but not limited to ovarian cancer, pancreatic cancer, melanoma, lymphoma, leukemia, and others. The tailored approach of PBCVs holds promise for personalized cancer immunotherapy across a wide spectrum of malignancies.

Conclusion: The Potential Benefits of Personalized Bacterial Cancer Vaccines (PBCVs) in the Promise of Curing Cancers

[0195] Cancer continues to be one of the most challenging diseases, affecting millions of lives worldwide. However, recent advancements in immunotherapy have opened up new avenues for targeted cancer treatment. Among these innovative approaches, Personalized Bacterial Cancer Vaccines (PBCVs) have emerged as a promising frontier. PBCVs offer a unique and tailored approach to cancer treatment by harnessing the power of the patient's immune system and leveraging the capabilities of genetically engineered bacteria. The benefits include, but are not limited to, the following:

[0196] 1. Personalized Medicine and Targeted Therapy. PBCVs represent a paradigm shift towards personalized medicine in cancer treatment. On the one hand, by analyzing the patient's genomic profile, PBCVs can identify neoantigens, which are specific to the individual and their tumor. On the other, by sampling the patient's microbiome, organisms that are safe and can colonize the patient's gastrointestinal tract can be identified.

[0197] This personalized approach enables the design of vaccines that precisely target the cancer cells, minimizing off-target effects and enhancing therapeutic efficacy. The ability to tailor treatment to the unique characteristics of each patient holds great potential for improving outcomes and minimizing adverse effects associated with traditional cancer treatments.

[0198] 2. Enhanced Immunogenicity and Anti-Tumor Immune Response. PBCVs have the ability to activate and enhance the patient's immune response against cancer cells. By expressing and secreting neoantigens, PBCVs effectively present these tumor-specific targets to the immune system, triggering a robust anti-tumor immune response. This immune activation can lead to the destruction of cancer cells locally and systemically, offering the potential for long-lasting and durable responses. PBCVs hold the promise of overcoming immune tolerance

mechanisms that often hinder conventional cancer treatments, such as immune checkpoint inhibitors.

[0199] 3. Broad Applicability and Versatility. One of the remarkable features of PBCVs is their potential applicability across various types of cancers. The ability to isolate and engineer bacteria, such as *E. coli*, *Bacillus* spp, or *LactoBacillus*, allows for targeting a wide range of solid tumors and hematological cancers. Moreover, PBCVs have shown promise in pediatric cancers, where conventional treatment options are often limited. This versatility makes PBCVs a potential therapeutic option for a broad spectrum of cancer patients, increasing the reach and impact of this novel approach.

[0200] 4. Targeting the Gut Microbiome-Immune Axis. The gastrointestinal tract houses a significant portion of the human immune system and plays a crucial role in regulating immune responses. PBCVs, when administered orally, can colonize the gut and interact with the gut microbiome, thereby modulating the local immune environment. This targeted approach takes advantage of the gut microbiome-immune axis to potentiate the immune response against cancer cells. The unique interplay between PBCVs, the gut microbiome, and the immune system holds tremendous potential for harnessing the power of the body's own defenses against cancer.

[0201] 5. Evolutionary Adaptability and Staying Ahead of Cancer Mutations. Cancers are notorious for their ability to evolve and develop resistance to conventional treatments. However, PBCVs have the potential to stay one step ahead of cancer mutations. By continually monitoring and analyzing the patient's tumor, PBCVs can adapt their neoantigen repertoire and target emerging cancer variants. This adaptability provides an advantage over static treatments and opens up possibilities for dynamic and personalized therapeutic interventions to counteract cancer's adaptive mechanisms.

[0202] The present invention provides a comprehensive and innovative approach for the development and utilization of Personalized Bacterial Cancer Vaccines (PBCVs) in the treatment of various cancers. By combining cutting-edge technologies in genomics, genetic engineering, bioinformatics, and immunotherapy, this patent outlines a method that holds immense promise for improving cancer outcomes and potentially achieving a cure.

[0203] Through the personalized nature of PBCVs, the invention addresses the inherent heterogeneity and complexity of cancer, allowing for tailored treatments that precisely target neoantigens unique to each patient's tumor. By leveraging the patient's immune system, PBCVs

stimulate a potent and specific anti-tumor immune response, leading to the destruction of cancer cells both locally and systemically.

[0204] The versatility of PBCVs is evident in their applicability to different types of cancers, including solid tumors, blood cancers, and pediatric cancers. Furthermore, by targeting the gastrointestinal tract and utilizing the gut microbiome-immune axis, PBCVs tap into a critical immune hub, enhancing the body's defense mechanisms against cancer.

[0205] The invention also encompasses the use of computational bioinformatics and artificial intelligence techniques to optimize the selection of neoantigens, ensuring the highest immunogenicity and therapeutic efficacy. Additionally, the patent describes robust manufacturing processes adhering to Good Manufacturing Practices (GMP) standards, ensuring the production of safe and quality PBCVs for clinical use.

[0206] Overall, the present disclosure provides a comprehensive roadmap for the development, manufacturing, and administration of PBCVs as a personalized and potent therapeutic approach for cancer treatment. By offering the potential to cure cancers, PBCVs have the capacity to transform the landscape of oncology, providing new hope for patients and revolutionizing the field of cancer immunotherapy.

[0207] In conclusion, the present invention represents a significant advancement in the field of cancer treatment, bringing us closer to the realization of personalized, effective, and curative strategies for combating this devastating disease.

EXAMPLES

[0208] Various aspects and advantages of the disclosure are illustrated by the following non-limiting examples.

Example 1: Isolation of *E. coli* and *Bacillus spp.* from Patient Stool Samples

[0209] The stool samples of a patient can be stored in the -20°C freezer until use. The samples can then be thawed at room temperature, separated into segments weighing 2 grams each, placed into tubes along with 2 grams of phosphate buffered saline at a 1:1 ratio and shaken to mix the contents.

[0210] To isolate *E. coli* from the sample, 100 uL of sample is directly plated on a 150 mm diameter MacConkey agar plate (Remel). Additional samples are also generated by adding 2

mL of lysogeny broth (Miller) to the remaining stool sample (~500 uL to 1 mL of sample) and allowed to incubate for 2 hours at 37°C to assist in *E. coli* recovery.

[0211] The sample is then centrifuged at 3000 rcf for 2 minutes and 50 uL of the pellet is plated on another MacConkey agar plate. When necessary, 10 and 100x dilutions are performed prior to plating to obtain single colonies. The plates are incubated overnight (~20 hours) at 37°C. Colonies exhibiting lactose-fermenting *E. coli* morphology on MacConkey agar are saved for further evaluation.

[0212] Various techniques are used to isolate the *Bacillus* spp. spores which included boiling, ethanol shock, or a combination of both. The procedure for boiling includes diluting the stool sample in phosphate buffered saline at a 1:1 ratio followed by heating at 80°C for 20 minutes. The procedure for ethanol shock includes mixing the intestinal contents with 95% ethanol at a 1:1 ratio and incubating at room temperature for 1 hour. If both selection methods are used, the ethanol shock is done first followed by the boiling.

[0213] After the selection process is completed, ~0.5mL of the treated stool contents are plated on various microbiological media such as Brain Heart Infusion agar, Luria-Bertani agar, Mueller-Hinton agar, and Nutrient Agar. Plates are incubated overnight at 37°C and individual colonies are isolated and transferred for subsequent testing and preservation.

[0214] Unless otherwise noted, all *E. coli* and *Bacillus* spp. after isolation were cultivated aerobically in Luria-Beranti broth at 37°C using a rotary shaker (200rpm) for 18-20 hours. When necessary, antibiotics were supplemented into the media for engineered derivatives and included chloramphenicol (20ug/mL) and kanamycin (75ug/mL) for *E. coli* and kanamycin (20ug/mL) for *Bacillus* spp.

Example 2: Experiments to establish isolate uniqueness

[0215] To verify that colonies on MacConkey plates are indeed *E. coli*, two independent colony Polymerase Chain Reaction (PCRs) are performed using primers that bind to the *gadA* and *gadB* promoter regions in the *E. coli* chromosome. PCRs were performed using GoTaq Green Mastermix with the parameters shown below. The primers, annealing temperatures, and expected band sizes are listed in Table 5.

[0216] *E. coli* Nissle 1917 as well as another verified *E. coli* isolate are used as positive controls. Detection of one or both PCRs with the expected band size signifies positive *E. coli*

identity. Five positive isolates from each segment of the patient's stools are grown overnight in lysogeny broth and saved as freezer stocks at -80°C.

[0217] PCR Conditions: Initial Denaturation: 95°C 2 minutes. Repeat 30x: 95°C 1 minute, 45°C 1 minute. 72°C 30 seconds. Final extension: 5 minutes

Table 5. Primers used to verify *E. coli* Identity

Gene	Primers (Forward and Reverse, 5'-3') (Derived from <i>Escherichia coli</i>)	Annealing Temp	Expected Amplicon Length (basepairs)
gadA	gadA F: GATCGCCCGAACAGCAATG (SEQ ID NO: 6)	45	174
	gadA R: ATAAAAAAGTAGGATTTATCCGC (SEQ ID NO: 7)		
gadB	gadB F: TCAAGTACTAATAGTGATATTTTAAGG (SEQ ID NO: 8)	45	242
	gadB R: TTAAAAAAGTAGGATTTATCGATAAAG (SEQ ID NO: 9)		

[0218] Prospective *Bacillus* spp. isolates that were morphologically distinct from one another were selected for DNA fingerprinting. In this procedure, a polymerase chain reaction was performed on the *Bacillus* colonies using BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3', SEQ ID NO: 37). The PCR conditions are described below. After the PCR was complete, the reactions were loaded onto a 1.5% agarose gel and run at 120V for two hours. Isolates that had similar band patterns were grouped together and a representative isolates from each group were selected for subsequent testing and preservation.

[0219] The PCR was performed using Promega GoTaq Green Mastermix according to the manufacturer's protocol. The PCR reaction mixture consisted of 12.5 uL of GoTaq Green Mastermix, 2 uL of the BOXA1R primer (10 µM solution), and 10.5 uL of autoclaved distilled water. A colony of *Bacillus* was swabbed with a sterile tip and placed into the reaction mixtures. The tip was then ground into the reaction tube to dislodge cells and release their DNA.

[0220] The thermocycler settings were as follows:

Initial denaturation 95°C for 10 minutes

35 cycles

95 °C for 30 seconds

50 °C for 30 seconds

72°C for 8 minutes

Final extension 72 °C for 5 min

Example 3: 16S Sequencing of the Isolates

[0221] *Bacillus* spp. isolates were sent for 16S rRNA sequencing to identify the species of the isolate. The 16S rRNA gene was amplified using polymerase chain reaction (PCR) with the universal primers, 27F (5'AGAGTTTGATCMTGGCTCAG-3', SEQ ID NO: 38) and 1492R (5'-GGYTACCTTGTTACGACTT-3', SEQ ID NO: 39). The reaction conditions are stated below. PCR reactions were visualized using gel electrophoresis and the expected band (located at ~1500 base pairs) was excised for each strain, gel-purified, and sent for Sanger sequencing through AGCT Inc. Species was estimated via standard sequence alignment of the resulting 16S rRNA sequences using the Basic Local Alignment Search Tool [Nucleotide BLAST (blast.ncbi.nlm.nih.gov/Blast)]. Through 16S sequencing GP1336 was initially identified as *Bacillus licheniformis* and GP1252 was identified as *Bacillus oleronius*. GP1336 (ATCC Accession No. PTA-127307) was later discovered to be a *Bacillus paralicheniformis* strain after the whole genome was sequenced.

[0222] The PCR was performed using Promega GoTaq Green Mastermix. The PCR reaction mixture consisted of 12.5 uL of GoTaq Green mastermix, 1 uL of each primer (10 µM solution), and 10.5 uL of autoclaved distilled water. A colony of *Bacillus* was swabbed with a sterile tip and placed into the reaction mixtures. The tip was then ground into the reaction tube to dislodge cells and release their DNA.

[0223] The thermocycler settings were as follows:

Initial denaturation 98°C for 10 minutes

35 cycles

98 °C for 30 seconds

55 °C for 30 seconds

72°C for 1 minute 30 seconds

Final extension 72 °C for 10 min

Example 4: Protease Activity of *Bacillus* spp. Against Neoantigen Peptides

[0224] Several attempts are made to express and secrete various known cancer neoantigens from *Bacillus subtilis* 168 without success. Standard troubleshooting with genetic manipulation suggested that the lack of production was not due to transcriptional or translational bottlenecks. Additionally, several secretion mechanisms were attempted.

[0225] It was questioned whether the strain may be producing but rapidly inactivating the peptides of interest. To test this hypothesis, cultures of *Bacillus subtilis* 168 were grown overnight and cells were removed by centrifugation. The resulting supernatant was then sterilized via filtration through a 0.22 μ m polyethersulfone filter.

[0226] *Bacillus subtilis* supernatant was then incubated with the peptides of interest and peptide presence was tested via electrophoresis. It was found that incubation with *B. subtilis* 168 supernatant for just 3 hours resulted in complete elimination of peptide activity.

[0227] Protease activity has been examined in the context of heterologous protein expression [Wu *et al.*, Applied and Environmental Microbiology, 68, 3261–3269 (2002)]. Suspected proteases in the genome of the producer strain have been knocked out, as in Thompson *et al.*, FEMS Microbiology Letters, 367, 162 (2020) which describes the 8-gene knockout *Bacillus subtilis* Δ 8–22. Strains with these genes knocked out have exhibited higher yields of other heterologous proteins [Wu *et al.*, *supra*].

[0228] *B. subtilis* Δ 8–22 was tested with several genetic constructs to produce peptides. However, no production was observed. Supernatant protease activity assays were performed with this strain. Despite the elimination of all detectable protease genes, rapid peptide inactivation was still observed. Thus, the heterologous production of peptides was unable to be attained using traditional methods previously known to those skilled in the art.

[0229] An alternative solution was identified in which the production strain would be selected through medium-throughput screening of natural isolates for compatibility with the peptide of interest. To *determine* compatibility, supernatant protease assays were performed for panels of *Bacillus* isolates and isolates exhibiting the lowest levels of protease activity or peptide inactivation were selected for genetic manipulation.

[0230] For the protease assay, selected isolates were inoculated into Brain Heart Infusion (BHI) medium and grown shaking at 37°C for ~20 hr. After the incubation, 2 mL of each *Bacillus* culture was transferred to 2mL conical tubes and centrifuged to remove cells (13,000xg, 1

minute). *Bacillus* spp. supernatant was then filter-sterilized using a 0.22 um polyethersulfone filter.

[0231] The peptide of interest can be produced using various strains of bacteria known to produce high quantities of the peptides. 50 mL cultures of the producer strains were grown in BHI at 37°C for ~20 hr. Cultures were then centrifuged to remove cells and supernatant was sterilized by boiling for 10 minutes at 100°C. The act of boiling at this time and temperature has been found to eliminate any protease activity from the producer supernatant without impacting peptide activity. This supernatant will be referred to as the “peptide stock.”

[0232] 500 uL of *Bacillus* supernatant was mixed 500 uL of the peptide stock and incubated at 37°C for 3 hours. 500 uL of plain BHI was mixed with 500 uL of the peptide stock as a negative control for protease activity. 500 uL of plain *B. subtilis* 168 supernatant was mixed with 500 uL of peptide stock as a positive control for protease activity. After the set time of incubation, 180 uL of the mixed supernatant was placed into sterile PCR tubes and boiled in the thermocycler (100°C, 10min) to inactivate protease activity.

[0233] A *Bacillus* isolate was considered to exhibit “favorable protease activity” if peptide detection techniques (e.g. mass spectrometry or protein gels) identified the peptide in the peptide/supernatant mixture. If no peptide could be observed, the isolate was considered unfavorable and was discounted for further assessment.

Example 5: Testing for Antibiotic Resistance of bacterial isolates

[0234] One of the primary concerns when considering any new probiotic is their resistance to antibiotics and their ability to transfer these resistance components to other microbes. All isolates are screened for antibiotic resistance against a panel of antibiotics. Screening was done by first growing the isolates overnight on *E. coli*-selective or BHI agar. Isolates were then struck onto *E. coli*-selective or BHI agar containing the antibiotic of interest.

[0235] Examples of antibiotics tested and their concentrations are spectinomycin (100ug/mL), kanamycin (20ug/mL) and erythromycin (0.5ug/mL).

[0236] Isolates were deemed favorable if they exhibited no observable growth on antibiotic plates.

Example 6: Testing for Hemolytic activity in isolates

[0237] Isolates are tested for hemolytic activity by streaking fresh patches of each isolate on to Blood Agar medium (Thermo Scientific TSA with Sheep blood, part number R01201). Plates are incubated overnight at 37°C. The following morning, plates are analyzed for signs of beta-hemolysis which includes a clear halo containing lysed red blood cells surrounding the bacterial growth. Isolates are selected when they show no signs of beta-hemolysis.

Example 7: Transformation of Isolates with Plasmids

[0238] For *E. coli* PBCVs, DNA constructs are first generated in standard cloning strains (ex. *E. coli* JM109 and *E. coli* MC1061 F'). Constructs are then isolated, sequence-verified, and transformed into the desired delivery host. *E. coli* isolated from patient stool samples are made electrocompetent using standard methods. Briefly, 100 mL of lysogeny broth (LB) is inoculated with 400 uL of an overnight culture of *E. coli* then incubated at 37°C under agitation until the culture reaches an OD600 of ~0.4. The cells are pelleted down by centrifugation at 4000xg for 10 minutes and washed three times in ice cold deionized water. The final pellet is resuspended in 400 uL ice cold 10% glycerol in deionized water.

[0239] For transformation, 50 uL of electrocompetent cells are thawed on ice then mixed with 50-200 ng of vector. *E. coli* are transformed at 2400 V, 25 uF, 200 Ohms in a 0.2 um gap cuvette. The transformation is then incubated in 400 uL SOC medium for 1 hr at 37°C and plated on LB agar containing the appropriate selective antibiotic.

[0240] Neoantigen peptide plasmid design is described in detail in Example 10. *Bacillus* PBCVa are transformed with the plasmid of interest via electroporation. Cells were made competent using the protocol described by Lu *et al.* [Letters in Applied Microbiology, 55, 9–14 (2012)]. This protocol was originally intended for transformation of *Bacillus subtilis* 168 derivatives but was found to be effective for various isolates of *Bacillus*.

Making electrocompetent stocks

[0241] *Bacillus* PBCVs are struck out on lysogeny broth (LB) plates and incubated overnight at 37°C. The next day, a colony is selected from each of the plates and is inoculated into a 3 mL LB culture and incubated overnight shaking at 37°C. The following day, 50 uL of the overnight culture is inoculated into 3mL of LB and incubated shaking at 37°C for 17 hrs. After the 17 hr incubation, 1 mL of the culture is inoculated into a 100 mL Erlenmeyer flask containing 40 mL of

LB + 0.5 M sorbitol. The flask culture is incubated shaking at 37°C until the 600 nm optical density (OD600) reaches ~ 0.8.

[0242] Once the cultures reach the correct OD600, the cultures are placed into 50 mL conical tubes and incubated on ice for 5 minutes. After the 5-minute incubation, the cultures are centrifuged at 5,000xg for 10 min at 4°C to collect the cells. After the cells are pelleted, 15 mL of ice-cold *Bacillus* electroporation buffer (0.5M sorbitol, 0.5M trehalose, and 0.5M mannitol in 10% glycerol) is dispensed into the tubes and the pellets are resuspended via shaking. Two additional wash cycles are performed in which cells are centrifuged at 5000-6,000xg for 10-20 minutes then resuspended in 15 mL electroporation buffer. After the wash cycles, the remaining pellet is resuspended with 500 uL of the electroporation buffer solution. 80 uL aliquots are made and stored at -80°C until use.

Electroporation of competent cells

[0243] For the transformation of electrocompetent isolates, an 80 uL aliquot of competent *Bacillus* cells are thawed on ice and 100 ug of plasmid are added to the cells. The cells are transferred to a 1mm cuvette and incubated on ice for 10 minutes. After the 10-minute incubation is complete, the cuvette containing the cells is placed in the electroporator. The settings were set at 2100V, 200 Ohms, and 25 uF and a single pulse is sent through the cells. The expected time constant is ~4.5ms.

[0244] After a successful pulse, the cells are recovered in 1mL of BHI containing 0.5M sorbitol and 0.38M mannitol shaking for 3 hours. After the 3-hour incubation, the cells are pelleted (5000xg, 5 minutes) and resuspended in ~100 uL of media. The cells are then plated on BHI plus kanamycin (20 ug/mL) and incubated overnight at 37°C.

[0245] Isolates that give colonies the next day are tested for the presence of the plasmid via colony PCR and/or using agar diffusion assays (see Example 14). The size of the transformant colonies varied depending on the isolate and the smaller colonies tended to not grow well. This decrease in growth and viability may indicate incompatibility of the plasmid system in those isolates.

Example 8: Generation of the Neoantigen Peptide (NAP) Plasmids pGP8001-3

[0246] Plasmids containing the neoantigen of interest along with any additional adjuvant genes were constructed using a seamless assembly method that employs digestions along with subsequent ligations in the same reaction. The method utilizes plasmids containing separate building blocks of a transcriptional unit (i.e. promoters, RBSs, genes, and terminators) that are isolated via digestion and ligated into a new plasmid backbone forming a functional transcriptional unit.

[0247] Plasmids used to modify *Bacillus* for the production of NAPs contained at minimum, the following components: 1) a plasmid backbone consisting of a *Bacillus*-compatible origin of replication, an *E. coli*-compatible origin of replication, and a selection marker (either antibiotic-resistance or auxotrophy-dependent); 2) one or more mature neoantigen peptides fused to one or more secretion tags; 3) promoter region; 4) ribosomal binding site; and 5) terminator.

[0248] Figure 2 depicts an example of a typical plasmid (pGP8001 (p43_ThyA)) used to produce a neoantigen peptide (in this case a Tp53-derived neoantigen, a peptide with 21 amino acids (amino acids 165-185 of the native p53 with a single mutation R175H) from *Bacillus* isolates. *Bacillus* replication gene (repB) and the pUB110 origin of replication comprise the replication components necessary to maintain a high plasmid copy number in the *Bacillus* spp. cell. The plasmid contains the *E. coli* pMB1-derived origin of replication necessary for cloning in *E. coli*. Additionally, an *E. coli*-compatible ampicillin resistance gene was included in preliminary plasmids to facilitate molecular cloning in *E. coli*. This resistance gene can be eliminated prior to the final application. The sequence of the entire plasmid is SEQ ID: 40.

[0249] Plasmid pGP8001 in Figure 2 (SEQ ID: 40) also includes an example of the transcriptional unit for the neoantigen peptide production as well as an example of the transcriptional unit for the auxotrophy-selection gene (thyA in this case). For the *Bacillus* spp. isolates that are not auxotrophs, a kanamycin resistance transcriptional unit is used in place of the thyA transcriptional unit.

[0250] To optimize peptide expression and isolate growth, numerous combinations of the above components were tested. Each component can impact the overall growth and production from the various isolates.

[0251] Figure 3 shows examples of possible neoantigen peptide transcriptional units. In one example, the promoter regulating the neoantigen peptide transcriptional unit is the *Bacillus*-

derived constitutive promoter, p43. In this example, the peptide of interest was a Tp53 neoantigen with a single missense mutation (R175H or G524A).

[0252] Figure 3 shows the use of the R0, R4, or R5 ribosomal binding sites and B0015 terminator sequence (SEQ ID NO: 5 [parts.igem.org/Part:BBa_B0015]) in the neoantigen peptide transcriptional unit. These components, particularly the ribosomal binding site, provide additional critical parameters to tune peptide expression. For example, Guiziou *et al.*, *supra* demonstrate that gene expression can be changed by orders of magnitude by altering the ribosomal binding sites.

[0253] Plasmids used to modify *E. coli* for the production of NAPs contained at minimum, the following components: 1) a plasmid backbone consisting an *E. coli*- compatible origin of replication and a selection marker (either antibiotic-resistance or auxotrophy-dependent); 2) one or more neoantigen peptides fused to the 3' end of a gene encoding an adjuvant protein or expressed on its own 3) promoter region; 4) ribosomal binding site; and 5) terminator.

[0254] Figure 4 depicts an example of a typical plasmid (pGP8002 (109_dapA) (SEQ ID NO: 41) used to produce a neoantigen peptide (in this case a Tp53-derived neoantigen, a peptide extended to 26 amino acids to include the methionine as the start codon (amino acids 160-185 of the native p53 with a single mutation R175H). The neoantigen peptide is codon-optimized for expression in *E. coli* and a part of an operon that also contains a gene encoding an adjuvant protein under the expression of the J23106 promoter. The plasmid contains an *E. coli* pMB1-derived origin of replication and the dapA gene under the expression of J23109 to act as the selective pressure in the dapA auxotroph derivative. In the absence of the dapA gene, *E. coli*-compatible chloramphenicol or kanamycin resistance genes can be utilized. The sequence of the entire plasmid is SEQ ID: 40 And utilizes the heat labile enterotoxin subunit B (eltB) as the adjuvant gene.

[0255] Plasmid pGP8003 in Figure 5 (SEQ ID: 42) contains the transcriptional unit that consists of a gene encoding the 21 amino acid segment of Tp53-derived neoantigen (amino acids 165-185 with a single mutation R175H) fused to the 3' prime end of the adjuvant gene. This fusion protein is expressed as one protein under the J23106 promoter. The sequence provided for the plasmid utilizes the eltB gene as the adjuvant. The ori and selective pressure of pGP8003 are identical to pGP8002 utilizing the dapA transcriptional unit to retain the plasmid. For the *E. coli* isolates that are not dapA auxotrophs, a kanamycin or chloramphenicol resistance transcriptional unit is used in place of the dapA transcriptional unit.

[0256] To optimize peptide expression and isolate growth, numerous combinations of the above components were tested. Each component can impact the overall growth and production from the various isolates.

[0257] Figure 6 shows examples of possible neoantigen peptide transcriptional units. In one example, the promoter regulating the neoantigen peptide transcriptional unit is the *E. coli* derived Anderson constitutive promoter, J23106. In this example, the peptide of interest was a Tp53 neoantigen with a single missense mutation (R175H or G524A).

[0258] Figure 6 shows the use of the BCD2 ribosomal binding site (SEQ ID NO: 45) and B0015 terminator sequence (SEQ ID NO: 5 [http://parts.igem.org/Part:BBa_B0015]) in the neoantigen peptide transcriptional unit.

BCD2 RBS (SEQ ID NO: 45)

gggccaagttcacttaaaaaggagatcaacaatgaaagcaattttcgactgaaacatcttaatcatgctaaggaggtttct

[0259] In addition to modifying the neoantigen peptide transcriptional unit, it was necessary to find an appropriate expression system for the auxotrophic selection marker. In Example 11 we describe construction of the dapA and thyA-based system. Figure 2 depicts the thyA gene under the control of the p43 promoter (SEQ ID NO: 40). Figures 4 and 5 depict the dapA gene under the control of J23109 promoter (SEQ ID NO: 12). However, it was found that growth, neoantigen expression, and plasmid stability could be tuned by implementing different promoters and ribosomal binding sites (see Figure 3).

Example 9: Generation of a *Bacillus* spp. isolate Secretion Tag Library

[0260] The commercially-available pBE-S plasmid (Mobitec) is modified to enable a peptide library expression system compatible with a diverse panel of *Bacillus* isolates.

[0261] To generate the new plasmid, the mature peptide of interest (lacking the secretion tag) is amplified and inserted into the NdeI restriction site of pBE-S using standard molecular cloning techniques. Next, the p43 promoter, R1, and a restriction site are inserted upstream of the peptide of interest using Gibson assembly. The p43 promoter and R1 RBS are selected because we had previously found them to be operational in various *Bacillus* spp. isolates.

[0262] The assembly is transformed into *E. coli* MC1061 F' and resulting colonies are verified using colony PCR and Sanger sequencing. A transformant containing the correctly assembled

plasmid is selected and cultured at 37°C overnight. The final assembled plasmid is then isolated using a standard miniprep protocol.

[0263] The plasmid miniprep is digested using MluI and EagI which removed a fragment directly upstream of the mature neoantigen peptide gene which would serve as the insertion location for the library of *Bacillus* secretion tags. The digest is run on a 0.8% agarose gel, excised, then gel purified.

[0264] The digested plasmid is then placed in a ligation reaction with the secretion tag mix (Takara Bio) using the In-Fusion HD Enzyme Mix (Takara Bio). The reaction uses a 2:1 insert to vector ratio and is incubated at 50°C for 15 minutes. After incubation, the ligation product is transformed in *E. coli* HST08 using heat shock. Transformants are plated on LB with ampicillin (100 ug/mL) and incubated at 37°C overnight.

[0265] The next day, the transformant colonies are resuspended with ~5mL of LB and miniprep. The plasmid secretion tag library is transformed into the competent *Bacillus* isolates and then plated on BHI with kanamycin (20 ug/mL) and incubated overnight.

[0266] The next day 100-200 transformants are tested to screen for relative peptide production.

Example 10: Introduction of Rifampicin Marker in Isolates

[0267] To assist in the enumeration of the probiotic during development, the producer strains are marked with resistance to rifampicin. This is done through selection of spontaneous mutants on growth agar containing the antibiotic of interest at the concentration of interest.

[0268] Briefly, a *Bacillus* PBCV strain is struck out on a BHI agar and is incubated overnight at 37°C. A fresh colony is inoculated in a 3mL BHI culture and incubated overnight under aerobic conditions at 37°C. The next day the culture is centrifuged (16,000xg, 30 sec) and resuspended in ~100uL of media. The bacterial resuspension is spread on BHI + rifampicin (150ug/mL) and incubated overnight at 37°C.

[0269] The following day, colonies are selected and tested for growth on BHI agar plates. Colonies exhibiting wild-type growth and morphology were then saved for long-term storage as -80°C freezer stocks.

Example 11: Construction of ThyA and DapA Knockouts in *E. coli* and *Bacillus spp.*

[0270] Generally, plasmids are inherently unstable and are lost from the engineered strain in the absence of pressure. Typically, plasmids contain an antibiotic resistance gene and are maintained in the engineered bacteria by applying the designated antibiotic to the growth medium. Thus, only cells that have maintained the plasmid are able to propagate.

[0271] The use of a dedicated antibiotic resistance gene is unacceptable for bacteria that will be used in human or animal health because of the risk of gene transfer. Additionally, the required application of an antibiotic during production may lead to increased production costs and risk of antibiotic contamination in the final product.

[0272] An alternative method for plasmid maintenance was implemented for selected *Bacillus* and *E. coli* isolates. In this method, an essential gene was removed from the chromosome and was placed on the plasmid of interest. Examples of essential genes used for this purpose include but are not limited to *dapA* (4-hydroxy-tetrahydrodipicolinate synthase), *thyA* (thymidylate synthase A), or homologues thereof. *DapA* and *thyA* are involved in diaminopimelic acid and thymidine synthesis respectively. The construction the new plasmids and the chromosomal modifications of the isolates is detailed below.

[0273] Related to the construction of the *E. coli* knockout strain, a plasmid-based CRISPR-Cas9 using the Cas9 protein derived from *Streptococcus pyogenes* (Jiang *et al.* 2015), The system consists of two different plasmids called the guide RNA (gRNA) and Cas9 plasmid. The gRNA plasmid contains the flanking regions of the target gene, either the *dapA* or *thyA* gene locus, as well as the gRNA for the target gene, ampicillin resistance gene, and pMB-1 derived origin of replication for selection and replication in *E. coli*. The Cas9 plasmid contains the genes encoding the Cas9 protein and the λ -red recombinase system under the control of an arabinose-inducible promoter as well as the gRNA to target the ori of the gRNA plasmid under the control of the lac promoter. The plasmid uses the kanamycin resistance marker and the pSC101 temperature sensitive origin of replication.

[0274] First, electrocompetent *E. coli* cells were transformed with the pCas plasmid and subsequently plated on LB + Kan (50ug/mL) and incubated overnight at 30°C. The next day the presence of the plasmid was confirmed using colony PCR and selected colonies used to make electrocompetent cells for the introduction of the gRNA plasmid.

[0275] Electrocompetent *E. coli*:pCas cells were prepared in a similar fashion to the previous *E. coli* cells in Example 7 with two key differences. Firstly, the cells were grown in the presence

of kanamycin (50ug/mL) and at 30°C. Secondly, prior to harvesting at the desired OD600, the cells inoculating with arabinose solution (50mM final concentration) and incubated for 15 minutes at 250rpm to induce the λ -red recombinase system. After harvesting, the cells were transformed with the gRNA plasmid containing the flanking regions for either the thyA and dapA gene. The transformations were plated on LB supplemented with kanamycin (50ug/mL) and ampicillin (100ug/mL) along with the essential nutrient (diaminopimolic acid (DAP) or thymidine (Thy) (50ug/mL)) and incubated at 30°C overnight. The next day the colonies were screened for the correct deletion of the target gene.

[0276] Upon verification of the knockout, curation of the gRNA plasmid was initiated by inducing expression of the gRNA scaffold on the pCas that would target the ori of the gRNA plasmid. LB cultures were prepared and supplemented with kanamycin and either DAP or Thy along with IPTG (1mM final concentration) and incubated shaking for 17 hours at 30°C. After incubation, dilutions were plated on LB, LB + Kan+ Amp+ nutrient, and LB + Kan+ nutrient and incubated overnight at 30°C. Colonies from the Kan + nutrient plate were tested for the presence of the gRNA via cPCR and colonies confirmed to no longer contain the gRNA plasmid proceeded to the pCas curation step.

[0277] Prospective colonies free of the gRNA plasmid were inoculated in LB + nutrient cultures and incubated shaking at 37-42°C for ~17 hours to inhibit replication of the pCas plasmid. The next day, dilutions were plated on LB + nutrient and LB + Kan+ nutrient and incubated at 37°C overnight. Colonies from the LB + nutrient plates were tested for the presence of the pCas plasmid via cPCR.

[0278] Related to the construction of the *Bacillus* knockout strain, a plasmid was assembled to contain the pKS1 backbone, a spectinomycin resistance gene, and flanking regions targeting the gene of interest. The pKS1 backbone contains a temperature sensitive origin of replication compatible with both *E. coli* and *Bacillus*. The plasmid was assembled and transformed in MC1061 F'. The strain was grown at 30°C under aerobic conditions in BHI +spectinomycin (100ug/mL). Note that cells were grown at 30°C rather than 37°C to enable plasmid replication.

[0279] After confirmation of proper assembly via colony PCR, the plasmid was miniprepmed and transformed into *Bacillus* (see Example 9 for protocol). After confirmation of the presence of the plasmid in the given strain, a colony was selected and grown in BHI + spectinomycin at 37°C shaking for 24 hours. The increase of temperature would inhibit replication of plasmid but the presence of spectinomycin would force the whole plasmid to integrate into the target gene locus. This is the first recombination step at the thyA gene locus.

[0280] After the 24hr incubation, the culture was serially diluted, and the 100-104 dilutions were plated on BHI agar + spectinomycin and incubated overnight at 37°C. The next day, colonies were tested for proper plasmid integration into the chromosome using colony PCR. Colonies exhibiting proper integration were inoculated into 3mL liquid BHI cultures and were incubated under aerobic conditions at 30°C for 24 hr. This second incubation period allowed for the second recombination step in which the plasmid backbone was excised from the chromosome. Thus the resulting knockout strain was left with only regions flanking the gene of interest.

[0281] After the 24 hr incubation period, the culture was serially diluted, and the 100-104 dilutions were plated on BHI + thymidine (50ug/mL) and trimethoprim (10ug/mL) for thyA knockouts and incubated at 37°C overnight. The trimethoprim assists in eliminating cells that revert back to the wild-type genotype.

[0282] The next day, prospective thymidine auxotroph colonies were confirmed via colony PCR of the or thyA gene locus. Isolate growth was also tested on BHI or thymidine + spectinomycin to confirm that the plasmid was excised from the chromosome and on BHI lacking or thymidine to confirm the isolates were in fact auxotrophic for the nutrient of interest.

Example 12: Viability of PBCV Isolates in Various Intestinal Tract-Mimicking Environments

[0283] Genetic modification of bacterial strains can often result in hindered growth. These fitness defects can be detrimental to both manufacturing and in vivo efficacy. It is therefore prudent to test the growth of modified strains compared to their unmodified counterparts in relevant growth conditions.

[0284] In this example, PBCV isolates are tested in rich media typically used in laboratory assays as well as media generated from intestinal contents from mice. As such, it is crucial that the modified strain be metabolically active given the nutrient in that region.

[0285] For the growth assays, isolates are struck out from freezers stocks onto BHI agar plates and incubated overnight at 37°C. The next day overnight cultures are made of the strains in 3 mL of BHI and incubated overnight shaking at 37°C.

[0286] The next day, the fresh cultures were used to inoculate cultures for the growth assay. Growth curve assays were performed in 96 well plate format and included three replicates of each growth condition. 300 uL of the medium of interest is added to the appropriate wells and is

seeded with 3 uL of fresh culture of isolated PBCVs to be tested. Growth curves can be monitored using a Biotek Epoch2 spectrophotometer with continuous shaking at 37°C. Optical density readings at 600 nm (OD600) readings are taken every 15 minutes for 20 hours.

[0287] The conditions tested included used either nutrient-rich media (BHI) or jejunal contents. The jejunal content media was made using filter-sterilized intestinal contents isolated from healthy mice that had been diluted 5X in phosphate-buffered saline. The contents were then further diluted 5X in M9 minimal media.

[0288] In addition to testing different media, varying concentrations of Tween 80 are tested (0, 0.025, 0.05, and 0.075%) to decrease co-aggregation in the wells. Tween 80 concentration help decrease the coaggregation and the cells do not seem to be strongly affected by the presence of the Tween 80 in the nutrient rich media. However, the Tween 80 seems to negatively impact the growth of the cells in the jejunal contents.

Example 13. Testing and screening of PBCVs in stomach-like environments

[0289] For PBCVs to be orally administered and delivered inside the gastrointestinal tract, it is beneficial that PBCVs survive passage through the acidic conditions of the stomach. PBCVs are thus tested for their sensitivity to stomach contents using survival assays in vitro. For these assays, contents are removed from the stomachs of healthy mice and are mixed with water (~1:1 ratio). The mixture is then centrifuged for 1 minute at 3500 rcf to remove large solids. The pH is measured for each experiment (pH ~2). Stomach contents are then inoculated with $\sim 10^7$ CFU/mL of each probiotic. The cultures are then incubated anaerobically at 37°C for 120 minutes. This time was selected as a conservative estimate of the residence time inside a patient's stomach.

[0290] The PBCV is enumerated at 0 hours, 1 hour and at 2 hours minutes by dilution plating on selective agar (BHI or LB+150 ug/mL rifampicin). Plates are incubated overnight at 37°C and the CFU/mL are determined for all time points. The survival ratio of each strain is then calculated as follows:

[0291] Survival Ratio = (CFU/mL after incubation) / (CFU/mL before incubation)

[0292] In addition to testing survival in stomach contents, survival of PBCVs is also tested in M9 minimal medium that had been adjusted to a pH of 2. This is done to test the impact of pH on the cells in the absence of any other variable biological components.

[0293] To be orally administered and delivered inside the gastrointestinal tract of patients, it is beneficial that PBCVs also survive passage through the bile of the duodenum. PBCVs are thus tested for their sensitivity to bile using survival assays in vitro. For these assays, contents are removed from the duodenum of healthy mice centrifuged for 1 minute at 3500 rcf to remove large solids. Duodenum contents are then inoculated with ~10⁷ CFU/mL of each probiotic. The PBCV cultures are then incubated anaerobically at 37°C for 3 hours. This time was selected as a conservative estimate of the residence time in a patient's duodenum.

[0294] The PBCV is enumerated at 0 hours and at 3 hours by dilution plating on selective agar (BHI or LB+150 ug/mL rifampicin). Plates are incubated overnight at 37°C and the CFU/mL are determined for both time points. The survival ratio of each strain is then calculated as follows:

[0295] $\text{Survival Ratio} = (\text{CFU/mL after incubation}) / (\text{CFU/mL before incubation})$

[0296] For PBCVs to be effective, they are preferably metabolically active inside the patient's intestinal tract. To test this parameter, mucus from the jejunum and ileum of healthy mice is isolated. The mucus is then diluted 10x in M9 minimal medium and sterile filtered to generate the biomatrix growth medium.

[0297] To perform the assay, the PBCV is first grown overnight in lysogeny broth (LB). 2.5 uL of the overnight culture is then added to 250 uL of the biomatrix growth medium in a sterile 96 well plate. The plate is then incubated at 37°C with agitation in a plate reader. Growth is monitored continuously for 20 hours based on optical density at 600 nm.

CLAIMS

1. A method for the development of Personalized Bacterial Cancer Vaccines (PBCVs) comprising:
 - a. collecting tissue samples from both normal tissue and cancer tissue of a cancer patient,
 - b. whole-genome sequencing of the tissue samples to identify DNA sequence differences,
 - c. identifying neoantigens, comprising tumor-specific DNA sequences, based on the DNA sequence differences between normal and cancer tissues,
 - d. isolating bacteria from the gastrointestinal tract of the patient and characterizing them as candidates for PBCVs,
 - e. genetically engineering selected bacteria to express and secrete the identified neoantigens, and
 - f. administering the recombinant PBCVs to the patient for cancer treatment.
2. The method of claim 1, wherein the bacteria used for genetic engineering are non-pathogenic and non-virulent.
3. The method of claim 1, wherein the bacteria used for genetic engineering are probiotics.
4. The method of claim 1, wherein the bacteria used for genetic engineering are isolated from the patient.
5. The method of claim 1, wherein the bacteria used for genetic engineering are *E. coli*.
6. The method of claim 1, wherein the bacteria used for genetic engineering are *Bacillus* spp.
7. The method of claim 1, wherein the bacteria used for genetic engineering are *LactoBacillus*.

8. The method of claim 1, wherein the neoantigens are carried by a plasmid in the bacteria.
9. The method of claim 1, wherein the neoantigens are integrated into the bacterial chromosome.
10. The method of claim 1, wherein one to twenty different neoantigens are encoded in the synthetic, polycistronic DNA.
11. The method of claim 1, wherein strong constitutive promoters are used to express the neoantigens, including the T7 promoter.
12. The method of claim 1, wherein inducible promoters are used to express the neoantigens, including the Lac promoter.
13. The method of claim 1, wherein the secretion of neoantigens is engineered and optimized using secretion tags.
14. The method of claim 1, further comprising in vitro testing of the recombinant microbe for expression and secretion of neoantigens.
15. A method of claim 1, wherein the immunogenicity of neoantigens is optimized through the use of a gene encoding a specific adjuvant.
16. A method of claim 1, further comprising the use of a specific adjuvant as either fused to or expressed as a separate protein from the neoantigens.
17. The method of claim 1, further comprising in vitro testing of the secreted neoantigens for immunogenicity.
18. The method of claim 1, wherein the recombinant PBCVs are administered orally.
19. The method of claim 1, wherein the recombinant PBCVs reach and colonize the gastrointestinal tract of the patient.

20. The method of claim 1, wherein the recombinant PBCVs release neoantigens in the lumen and epithelium of the small intestinal tract or the large intestine.

21. The method of claim 1, wherein an immune response is mounted against cancer cells that express the neoantigens.

22. The method of claim 1, wherein the recombinant PBCVs are effective in treating solid cancers.

23. The method of claim 1, wherein the recombinant PBCVs are effective in treating blood cancers.

24. The method of claim 1, wherein the recombinant PBCVs are effective in treating pediatric cancers.

25. The method of claim 1, wherein the recombinant PBCVs are personalized based on the specific neoantigens of each patient and based on bacteria isolated from the patient's gut microbiome.

26. The method of claim 1, wherein the recombinant PBCVs are effective due to optimal neoantigen selection.

27. The method of claim 1, wherein the recombinant PBCVs target the gut microbiome-immune axis to potentiate the immune response against cancer.

28. The method of claim 1, wherein the recombinant PBCVs can be used for treating, controlling, and preventing cancers.

29. The method of claim 1, wherein the recombinant PBCVs can be engineered to anticipate and target cancer cell mutations.

30. The method of claim 1, further comprising the step of manufacturing the recombinant PBCVs.

31. The method of claim 28, wherein the manufacturing process adheres to Good Manufacturing Practices (GMP) standards.

32. The method of claim 28, wherein the manufacturing process includes quality assurance and quality control (QA/QC) documentation.

33. A pharmaceutical composition comprising the recombinant PBCVs and a pharmaceutically acceptable carrier.

34. The pharmaceutical composition of claim 31, wherein the carrier is suitable for oral administration.

35. The pharmaceutical composition of claim 31, wherein the carrier is suitable for rectal administration.

36. A method for obtaining regulatory approval to administer the recombinant PBCVs to a cancer patient, comprising:

- a. submitting preclinical data demonstrating the safety and efficacy of the recombinant PBCVs,
- b. conducting clinical trials to assess the safety and efficacy of the recombinant PBCVs,
- c. submitting the clinical trial data to regulatory authorities for evaluation, and
- d. obtaining regulatory approval for administering the recombinant PBCVs to the cancer patient.

37. The method of claim 34, wherein the regulatory approval is obtained from the U.S. Food and Drug Administration (FDA).

38. A method of treating a cancer patient using the recombinant PBCVs, comprising:
a. administering a daily dose of 0.001, 0.01, 0.05, 0.1, or 0.2, or 0.3, or 0.4, or 0.5, or 0.6, or 0.7, or 0.8, or 0.9, or 1 billion CFUs of the recombinant PBCVs orally for a specified duration;

b. administering a daily dose of 0.1, or 0.2, or 0.3, or 0.4, or 0.5, or 0.6, or 0.7, or 0.8, or 0.9, or 1, or 2, or 3, or 4, or 5, or 6, or 7, or 8, or 9, or 10 trillion CFUs of the recombinant PBCVs orally for a specified duration;

c. administering a daily dose of 0.1, or 0.2, or 0.3, or 0.4, or 0.5, or 0.6, or 0.7, or 0.8, or 0.9, or 1 billion CFUs of the recombinant PBCVs rectally for a specified duration; or

d. administering a daily dose of 0.1, or 0.2, or 0.3, or 0.4, or 0.5, or 0.6, or 0.7, or 0.8, or 0.9, or 1, or 2, or 3, or 4, or 5, or 6, or 7, or 8, or 9, or 10 trillion CFUs of the recombinant PBCVs rectally for a specified duration.

39. The method of claim 36, wherein the specified duration is once, twice or thrice a day.

40. The method of claim 36, wherein the specified administration is daily for one day, multiple days, or continuous, indefinite daily administration.

41. The method of claim 36, wherein the specified administration is weekly for one week, multiple weeks, or continuous administration are prescribed intervals.

42. A method of validating the presence of the recombinant PBCVs in the gastrointestinal tract of a patient, comprising:

- a. collecting stool samples from the patient, and
- b. conducting PCR analysis to detect the presence of recombinant DNA specific to the PBCVs in the stool samples.

43. The method of claim 40, further comprising sequencing the detected recombinant DNA to confirm the presence of the PBCVs.

44. A method of assessing the immune response against cancer in a patient receiving the recombinant PBCVs, comprising:

- a. collecting blood samples from the patient, and
- b. analyzing the blood samples to measure the levels of immune cells, cytokines, and tumor-specific antibodies associated with anti-tumor immune response.

45. The method of claim 42, wherein the levels of immune cells, cytokines, and tumor-specific antibodies are measured using ELISA.

46. The method of claim 42, wherein the levels of immune cells, cytokines, and tumor-specific antibodies are measured using flow cytometry.

47. The method of claim 42, wherein the levels of immune cells, cytokines, and tumor-specific antibodies are measured using quantitative PCR.

48. The method of claim 42, wherein the levels of immune cells, cytokines, and tumor-specific antibodies are measured using Western blot analysis.

49. A method of assessing the efficacy of the recombinant PBCVs in inducing tumor regression or remission, comprising:

- a. monitoring the size and progression of tumors in the patient over a specified period, and
- b. analyzing tumor biopsy samples to assess the presence of cancer cells and the extent of tumor regression or remission.

50. The method of claim 47, wherein the tumor biopsy samples are subjected to histopathological examination.

51. The method of claim 47, wherein the tumor biopsy samples are subjected to immunohistochemistry analysis.

52. The method of claim 47, wherein the tumor biopsy samples are subjected to genetic sequencing to assess the presence of neoantigens and cancer-related mutations.

Figure 1

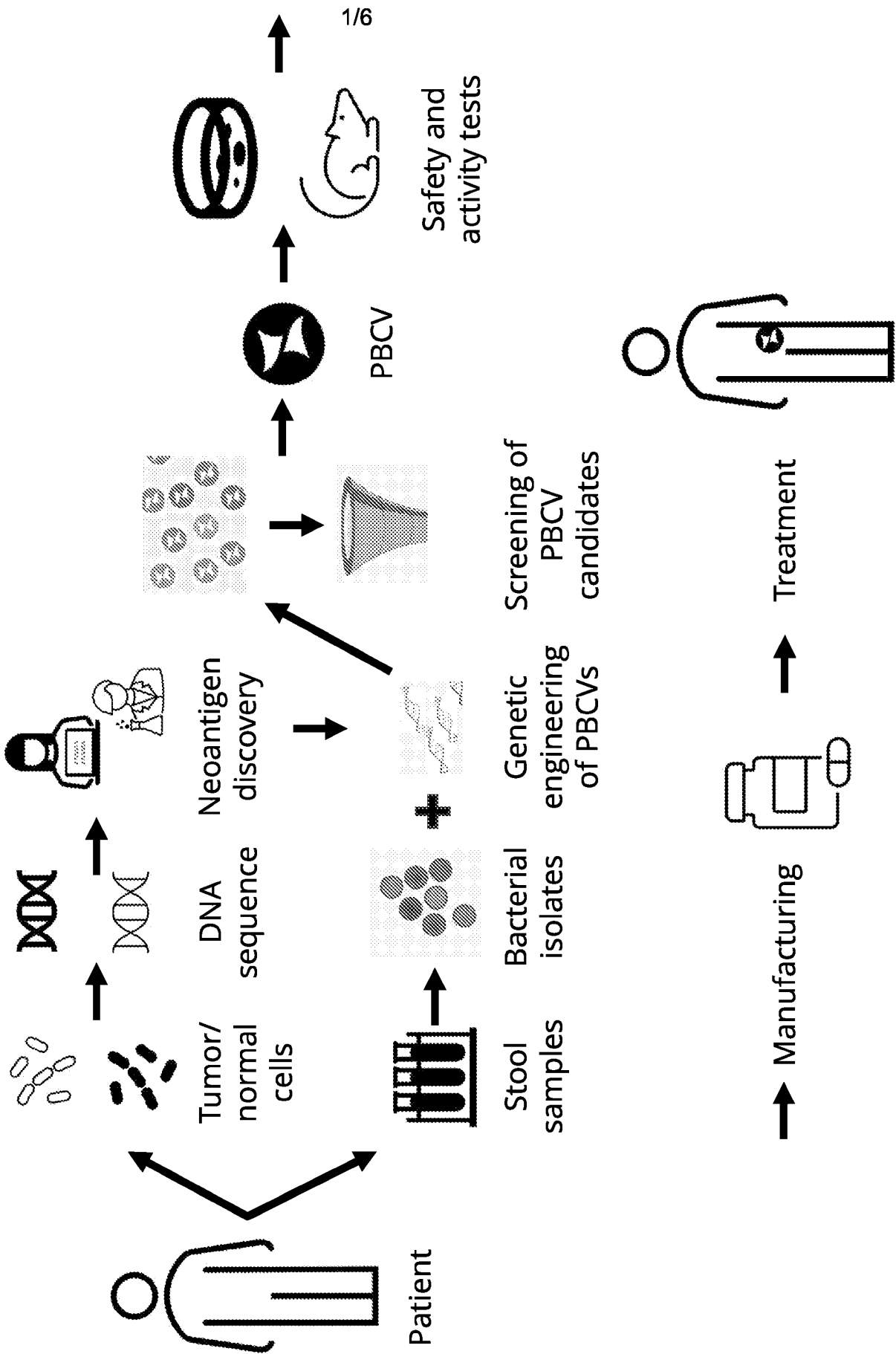


Figure 2



Figure 3

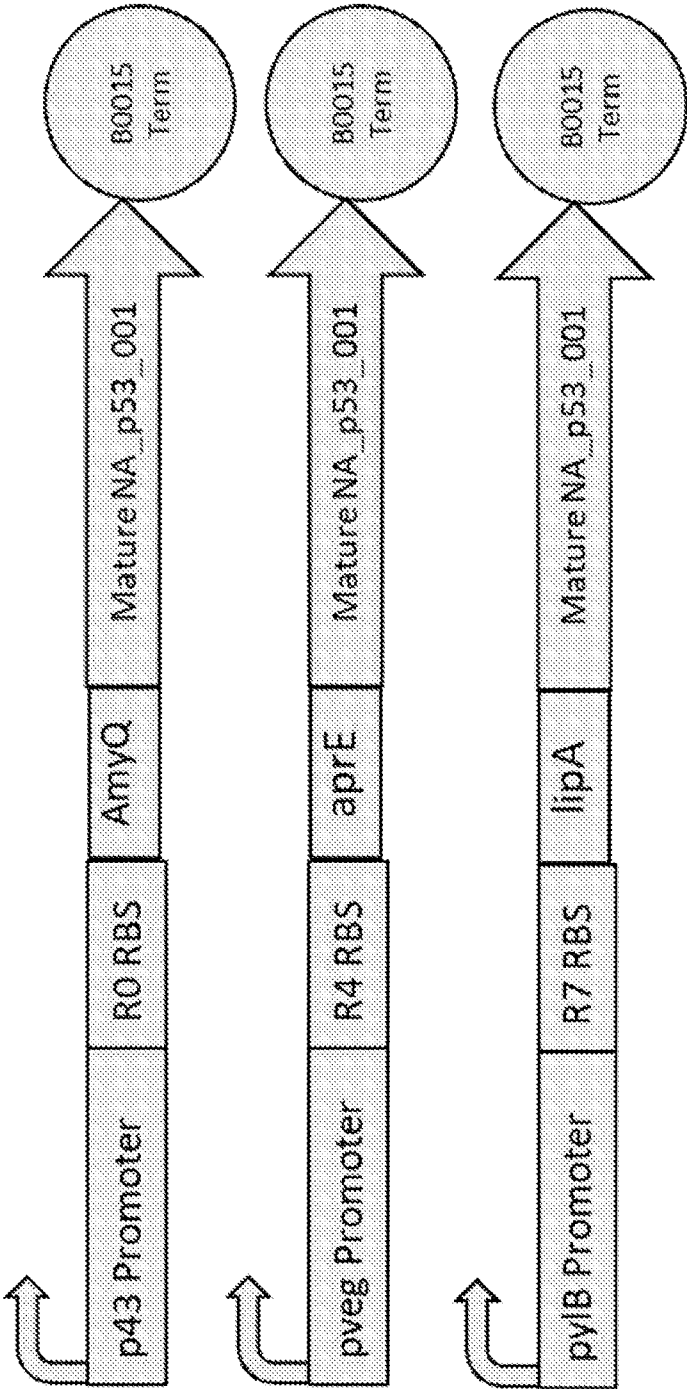


Figure 4

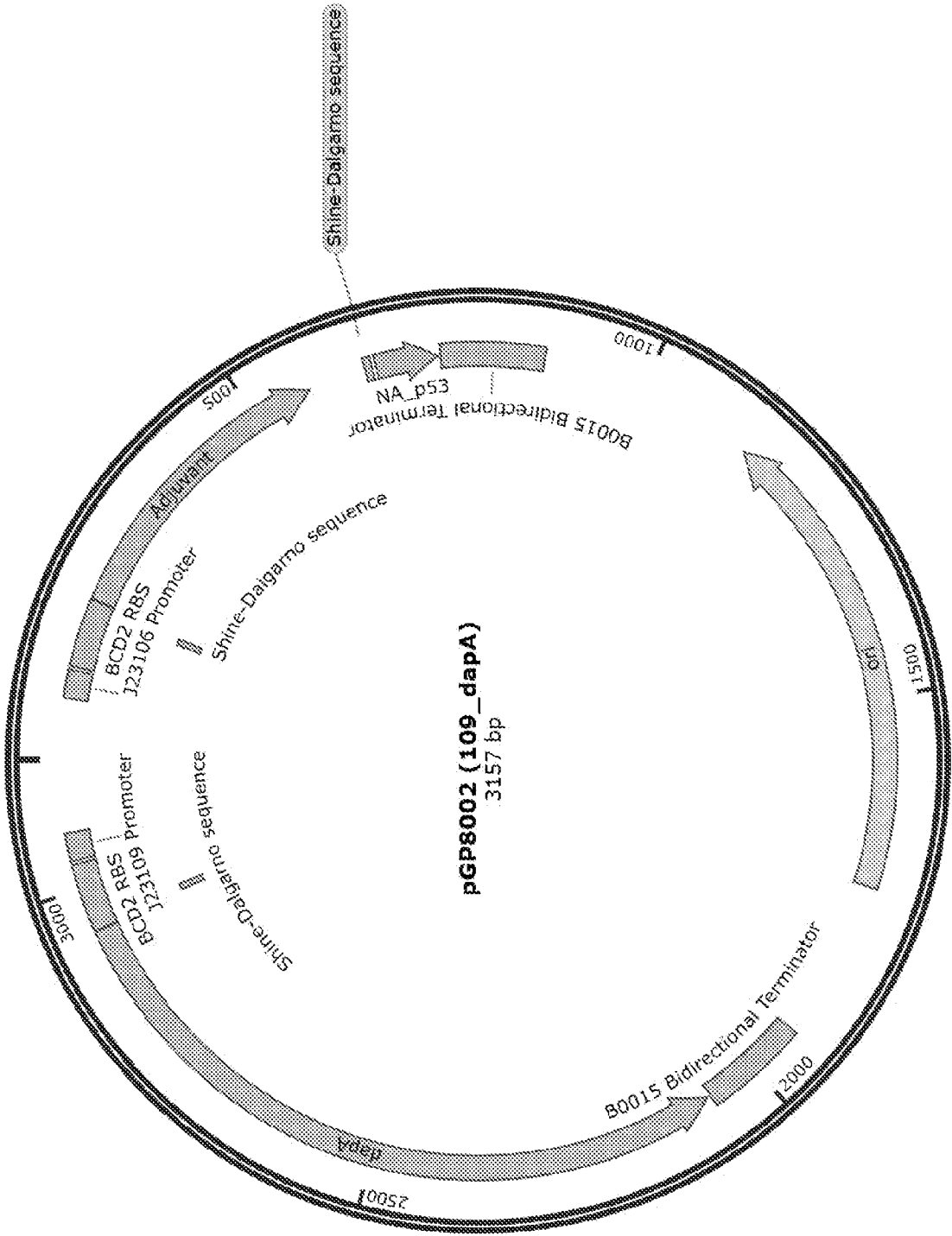


Figure 5

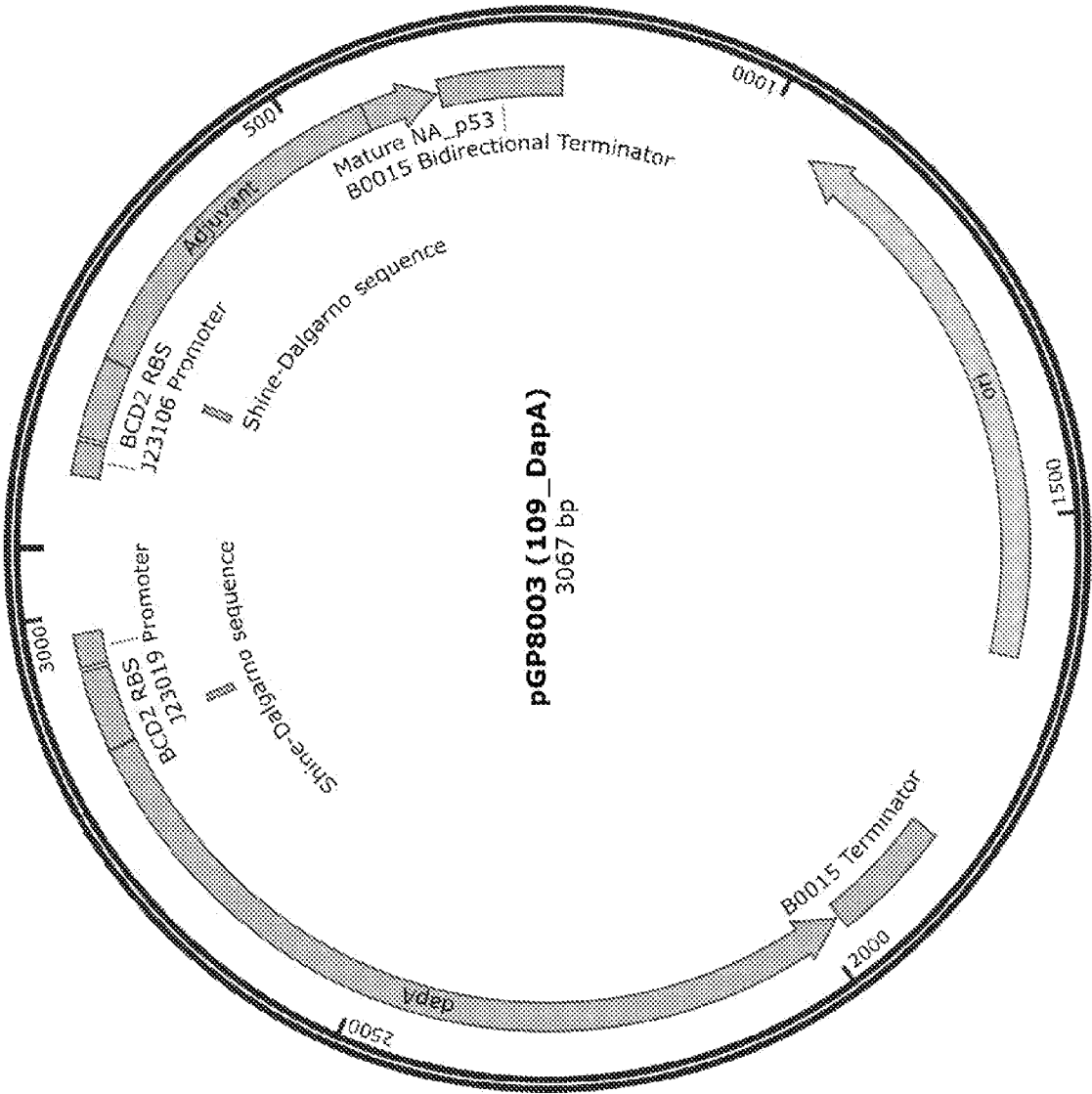


Figure 6

