

GENETICALLY MODIFIED BACTERIA HAVING STABLE MUTATIONS WITHOUT
THE REQUIREMENT OF ANTIBIOTIC MARKERS, AND SYSTEMS AND
METHODS FOR GENERATING SAME

CROSS-REFERENCE TO RELATED APPLICATION

5 [0001] The present application claims the benefit of U.S. provisional patent application serial number 63/622,157 filed on January 18, 2024. The contents of the above-referenced document are incorporated herein by reference in their entirety.

TECHNICAL FIELD

[0002] This application generally relates to the field of genetically modified bacteria
10 having stable mutations without the requirement of antibiotic markers, and to systems and methods for generating same.

SEQUENCE LISTING

[0003] The present specification is filed along a Sequence Listing in electronic format. The Sequence Listing file, entitled PhoenixSeqListing.xml, was generated on January
15 9, 2025 and is 83 kb in size. The information in electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

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BACKGROUND

[0005] The use of live, attenuated bacteria in medical applications has garnered
25 significant interest over the past decades, particularly in the fields of vaccine development and cancer therapy. Attenuated bacteria are strains that have been modified to reduce their virulence, ensuring safety while retaining their ability to interact with the host's immune system. These bacteria serve as efficient platforms for delivering antigens or therapeutic molecules, taking advantage of their natural
30 ability to infect and proliferate within host tissues.

[0006] In the realm of vaccine development, attenuated bacteria have been extensively employed as vectors for expressing heterologous antigens. Their capacity to induce immune responses, both systemic and mucosal, has positioned them as a versatile alternative to traditional vaccine platforms.

5 [0007] Beyond vaccines, attenuated bacteria are emerging as promising therapeutic delivery systems for the treatment of various pathologies, such as cancer treatment. For example, Bacterial replication in tumors can activate the immune system to recognize the tumor cells as foreign and initiate cytotoxic T cells to kill the cancer. Certain bacterial strains naturally home to and proliferate within tumor
10 microenvironments, exploiting the hypoxic and immunosuppressive conditions commonly found in solid tumors. Genetic engineering has further expanded their potential, enabling them to deliver therapeutic agents, such as cytokines, cytotoxic proteins, or immune checkpoint inhibitors, directly to the tumor site. This targeted approach not only enhances the efficacy of the treatment but also minimizes systemic
15 toxicity.

[0008] Despite these advancements, the practical application of attenuated bacteria in cancer therapy and as vaccine vectors faces several challenges. These include ensuring the safety and stability of the attenuated bacteria strains, achieving consistent colonization or delivery to the target site, and eliciting an appropriate
20 immune response. Additionally, there remains a need for further innovation to enhance the specificity, payload capacity, and immunogenic properties of these bacterial systems as well as reduce the potential for reversing an attenuated live vaccine strain to become virulent.

[0009] In light of the above, there is a need to provide attenuated bacteria having
25 stable mutations with reduced risk for the regain of virulence, thereby enhancing their safety and reliability for use in medical applications.

SUMMARY

[0010] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not
30 intended to identify key aspects or essential aspects of the claimed subject matter.

[0011] In a broad non-limiting aspect, the present disclosure relates to a composition comprising genetically modified bacteria, wherein the genetically modified bacteria comprise: a chromosomal disruption in an auxotrophic gene and in one or more

gene(s) involved in phosphate regulation, and a DNA rescue molecule comprising a functional copy of the auxotrophic gene, wherein the genetically modified bacteria are obtained from virulent pathogenic bacteria containing a virulence plasmid.

5 [0012] In a broad non-limiting aspect, the present disclosure relates to a method for eliciting an immune response in a host, the method comprising administering to the host an effective amount of the composition as described herein.

10 [0013] In a broad non-limiting aspect, the present disclosure relates to a method for preparing a live attenuated vaccine from virulent pathogenic bacteria containing a virulence plasmid, comprising causing a chromosomal disruption in the virulent pathogenic bacteria, the chromosomal disruption being in an auxotrophic gene and in one or more gene(s) involved in phosphate regulation, and introducing in the bacteria a DNA rescue molecule comprising a functional copy of the auxotrophic gene.

15 [0014] In a broad non-limiting aspect, the present disclosure relates to a vaccine comprising the composition as described herein and a pharmaceutical or veterinary-acceptable excipient, diluent, or carrier, for prevention of a bacterial infectious disease.

[0015] In a broad non-limiting aspect, the present disclosure relates to a method for delivering a therapeutic payload in a cancer treatment, comprising administration of the composition as described herein to a host having cancer, wherein the DNA rescue molecule further includes a recombinant gene coding for the therapeutic payload.

20 [0016] All features of exemplary embodiments that are described in this disclosure and are not mutually exclusive can be combined with one another. Elements of one embodiment can be utilized in the other embodiments without further mention. Other aspects and features of the present invention will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments in
25 conjunction with the accompanying Figures.

BRIEF DESCRIPTION OF DRAWINGS

[0017] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

30 [0018] A detailed description of specific exemplary embodiments is provided herein below with reference to the accompanying drawings in which:

[0019] FIG. 1A is a non-limiting illustration depicting the relative chromosomal localization of the *pst* operon and *glmS* gene (top) in *E. coli* and a strategy to disrupt (through deletion) the *pst* and *glmS* genes (bottom), in accordance with some embodiments of the present disclosure.

5 [0020] FIG. 1B is a non-limiting illustration depicting a system which includes disrupted chromosomal *pst* operon and *glmS* gene, and a plasmid vector and cassette each including a respective functional *glmS* gene, in accordance with some embodiments of the present disclosure.

[0021] FIG. 2 is a non-limiting colored illustration depicting plasmid map of pIJ355,
10 in accordance with some embodiments of the present disclosure.

[0022] FIG. 3A to FIG. 3D are non-limiting colored pictures of recombinant *E. coli* grown on various Luria–Bertani (LB) agar plates. The recombinant *E. coli* includes disrupted chromosomal *pst* operon and *glmS* gene, in accordance with some embodiments of the present disclosure. FIG. 3A: LB agar plate. FIG. 3B: LB agar
15 plate supplemented with N-acetyl glucosamine (NAG) (250 µg/ml). FIG. 3C: LB agar plate supplemented with 5-bromo-4-chloro-3-indolylphosphate di-sodium (BCIP) (50 µg/ml). FIG. 3D: LB agar plate supplemented with BCIP (50 µg/ml) and NAG (250 µg/ml). This growth pattern demonstrates that chromosomal disruption of *pst* operon and *glmS* gene renders the bacteria auxotrophic for NAG, and is identifiable
20 as a blue colony in media supplemented with a chromophore substrate.

[0023] FIG. 4 is a non-limiting colored illustration depicting the plasmid map of pIJ346, in accordance with some embodiments of the present disclosure.

[0024] FIG. 5 is a non-limiting colored illustration depicting the plasmid map of pIJ379, in accordance with some embodiments of the present disclosure.

25 [0025] FIG. 6A to FIG. 6D are non-limiting colored pictures of genetically modified *E. coli* grown on various LB agar plates. The genetically modified *E. coli* is obtained from the *E. coli* of FIG. 3A-3D, which has been transformed to contain the auxotrophic rescue *glmS* gene on a recombinant pAPEC-1 plasmid, in accordance with some embodiments of the present disclosure. FIG 6A: LB agar plate supplemented with
30 BCIP (50 µg/ml). FIG. 6B: LB agar plate supplemented with NAG (250 µg/ml). FIG. 6C: LB agar plate supplemented with BCIP (50 µg/ml). FIG. 6D: LB agar plate supplemented with BCIP (50 µg/ml) and NAG (250 µg/ml). This growth pattern

demonstrates that the rescue *glmS* gene on the plasmid complements the chromosomal disruption of the *glmS* gene.

[0026] FIG. 7A is a non-limiting picture of PCR products confirming disruption of *glmS-pstSCA* genes in a genetically modified *E. coli* bacteria sample, in accordance with some embodiments of the present disclosure. The ladder used was the Thermoscientific™ GeneRuler 1 kb Plus DNA Ladder Cat: SM1331.

[0027] FIG. 7B is a non-limiting illustration of a genetically modified *E. coli* bacterium of FIG. 7A, in accordance with some embodiments of the present disclosure. This bacterium is shown as still expressing O antigens, fimbriae, LPS, and the like, which are common antigens recognized by the immune system in response to infection.

[0028] FIG. 8A to FIG. 8C are non-limiting graph representations of bacterial counts present in the blood of vaccinated with genetically modified O7 APEC ($\Delta glmS$ -*pstSCA*- pAPEC-1 $\Delta iucABCD$ $\Delta iroBCDE::glmS$) and non-vaccinated chickens at 6-, 24-, and 48-hours post-challenge with virulent O7 APEC, in accordance with some embodiments of the present disclosure. The results are reported as colony-forming units (CFU) per milliliter of blood. Dots represent individual values for the CFUs in the blood of each chicken. The black line = the median level of colonization. Statements of statistical significance were based on the level of $P \leq 0.05$. Statistical differences were evaluated for pairwise comparisons between vaccinated and unvaccinated groups using a non-parametric Mann-Whitney test.

[0029] FIG. 9A to FIG. 9C are non-limiting graph representations of bacterial counts present in the organs (lungs, spleens, and livers) of chickens vaccinated with genetically modified O7 APEC ($\Delta glmS$ -*pstSCA*- pAPEC-1 $\Delta iucABCD$ $\Delta iroBCDE::glmS$) and non-vaccinated chickens at 48 hours post-challenge with virulent O7 APEC, in accordance with some embodiments of the present disclosure. The results are reported as CFU per gram of tissue. Dots represent individual values for the CFUs in each organ of each chicken. The black line = the median level of colonization. Statements of statistical significance were based on the level of $P \leq 0.05$. Statistical differences were evaluated for pairwise comparisons between vaccinated and unvaccinated groups using a non-parametric Mann-Whitney test.

[0030] FIG. 10 is a non-limiting colored scatter graph representation of the production of inflammatory lesions in air sacs of chickens vaccinated with genetically modified O7 APEC ($\Delta glmS$ -*pstSCA*- pAPEC-1 $\Delta iucABCD$ $\Delta iroBCDE::glmS$) and non-

vaccinated chickens at 48 hours post-challenge with virulent O7 APEC, in accordance with some embodiments of the present disclosure. Vaccination was performed with administration of the vaccine in the air sacs. Each dot represents the lesion score of an individual bird. Bars represent the mean from lesion scores of several birds per group. Statements of statistical significance were based on the level of $P \leq 0.05$. Statistical differences were evaluated for pairwise comparisons between vaccinated and unvaccinated groups using a non-parametric Mann-Whitney test.

[0031] FIG. 11 is a non-limiting colored graph representation of the percentage of weight differences between chickens vaccinated with genetically modified O7 APEC ($\Delta glmS$ - $pstSCA$ - $pAPEC$ -1 $\Delta iucABCD$ $\Delta iroBCDE::glmS$) and non-vaccinated chickens calculated at 24- and 48-hours post-challenge with virulent O7 APEC, in accordance with some embodiments of the present disclosure. Dots represent individual values for the weight of chickens. Bars represent the mean of weight changes of survival birds per group. Vertical lines above the bars represent the standard deviation. Statements of statistical significance were based on the level of $P \leq 0.05$. Statistical differences were evaluated for pairwise comparisons between vaccinated and unvaccinated groups using a non-parametric Mann-Whitney test.

[0032] FIG. 12 is a non-limiting colored Kaplan-Meier survival analysis of mice following challenge with wild type (WT) *Salmonella enterica typhimurium* SL1344 strain (QT1573) and with the genetically modified *Salmonella enterica typhimurium* SL1344 strain $\Delta glmS$ - $\Delta pstSCA$ - $pSLT::glmS^+$ (QT6282) in accordance with some embodiments of the present disclosure. All the mice were monitored daily for six days post-challenge for mortality. The mortality rate was 60% for the WT strain, and no mortality in the group infected with the mutated, genetically modified strain.

[0033] FIG. 13A to 13C are non-limiting colored graph representation of bacterial counts present in respective liver, spleen, and caecum of infected C57BL/6 mice. The mice were infected with 6.10×10^7 CFU of WT *Salmonella enterica typhimurium* SL1344 strain (QT1573), and 9.10×10^7 CFU of genetically modified *Salmonella enterica typhimurium* SL1344 strain $\Delta glmS$ - $\Delta pstSCA$ - $pSLT::glmS^+$ (QT6282), in accordance with some embodiments of the present disclosure. The results are reported as CFU per g of organ. Results are shown as mean $\pm$ SEM counted CFU per organ weight. Statistical differences were evaluated for pairwise comparisons between the group challenged with WT QT1573 and the groups challenged with mutant, genetically modified *Salmonella enterica typhimurium* SL1344 strain $\Delta glmS$ - $\Delta pstSCA$ - $pSLT::glmS^+$ (QT6282) using non-parametric Kruskal-wallis test.

[0034] FIG. 14A and FIG. 14B are non-limiting colored pictures of various *Salmonella enterica typhimurium* SL1344 bacteria clones lacking *glmS* and *pstSCA*. The clones failed to grow on M9 media without NAG and with BCIP (50 µg/mL) (FIG. 14A), but grew well on M9 media with NAG (250 µg/mL) and with BCIP (50 µg/mL) (FIG. 14B),
5 in accordance with some embodiments of the present disclosure. This growth pattern confirms that the clones have a disrupted *glmS* gene and are thus auxotrophic for NAG. A single clone was deposited in each quadrant of the M9 plate.

[0035] FIG. 15 is a non-limiting colored picture of a genetically modified *Salmonella enterica typhimurium* SL1344 bacteria sample (QT6282) lacking *glmS* and *pstSCA*,
10 and which includes a plasmid that contains a functional *glmS* gene, grown on M9 media without NAG and with BCIP (50 µg/mL), in accordance with some embodiments of the present disclosure. This growth confirms that the plasmid *glmS* gene rescues the disrupted *glmS*.

[0036] FIG. 16 is a non-limiting picture of PCR products that confirm the deletion of
15 the *glmS* and *pstSCA* gene in the genome of *Salmonella enterica typhimurium* SL1344 (SL1344 Δ *glmS* Δ *pstSCA*) and of *Salmonella enterica typhimurium* SL1344 (QT6282), in accordance with some embodiments of the present disclosure. The ladder used was the ThermoScientific™ GeneRuler DNA Ladder Mix SM0331.

[0037] FIG. 17 is a non-limiting picture of PCR products from *Salmonella enterica typhimurium* SL1344 clones to confirm the genetic stability of the *glmS*-*pstSCA* deletion and *glmS* insertion into the pSLT plasmid, after 5 passages on agar plates,
20 in accordance with some embodiments of the present disclosure. Using primers CMD3082/3083, a 2297 bp band was detected in both the SL1344 Δ *glmS* Δ *pstSCA* and QT6282 strains, confirming that the deletions are stable. Using primers
25 CMD3297/3298, a 1212 bp band was detected in the WT strain SL1344 and the mutant, genetically modified strain SL1344 Δ *glmS* Δ *pstSCA*, confirming the absence of *glmS* in the pSLT plasmid. A 3981 bp band was observed in the QT6282 strain after one passage on agar plates and after five passages, confirming the maintenance of the *glmS* insertion in the pSLT plasmid. The ladder used was the ThermoScientific™
30 GeneRuler DNA Ladder Mix SM0331.

[0038] FIG. 18 is a coloured picture of LB liquid media without NAG and with BCIP (50 µg/mL) tubes incubated at 37°C for 16 hours, as follows: (A) control – no bacteria inoculum, (B) wild type virulent pathogenic APEC, and (C) genetically modified *E. coli* which is based on the virulent pathogenic APEC in (B). This demonstrates that

genetically modified bacteria of the present disclosure can be optically detected through coloring of growth media containing a chromophore marker.

[0039] FIG. 19 is a non-limiting picture of PCR products to confirm the stability of the *glmS* inserted in the pAPEC-1 of the O7 mutated strain ($\Delta glmS$ -*pstSCA*- pAPEC-1
5 $\Delta iucABCD \Delta iroBCDE::glmS$) genome. Using primers *iroB_glmS_FW* (7) and *iroE_glmS_Rv* (8), a 2348 bp band was detected in both mutated O7 strain during the 10 passages in agar, confirming the maintenance of the *glmS* insertion in the pAPEC-1 plasmid. The ladder used was the GeneRuler Ladder Mix (SM0331). D= day.

[0040] In the drawings, exemplary embodiments are illustrated by way of example. It
10 is to be expressly understood that the description and drawings are only for the purpose of illustrating certain embodiments and are an understanding aid. They are not intended to be a definition of the limits of the invention.

DETAILED DESCRIPTION

[0041] The present technology is explained in greater detail below. This description
15 is not intended to be a detailed catalog of all the different ways in which the technology may be implemented, or all the features that may be added to the instant technology. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations
20 and additions to the various embodiments suggested herein will be apparent to those skilled in the art, considering the instant disclosure, which variations and additions do not depart from the present technology. Hence, the following description is intended to illustrate some embodiments of the technology, and not to exhaustively specify all permutations, combinations, and variations thereof.

[0042] The present inventors have, through R&D work, surprisingly and unexpectedly
25 developed a composition comprising genetically modified bacteria, which are attenuated bacteria having reduced risk to regain virulence. For example, the present inventors have developed a system and methods for generating and retaining gene disruptions in virulent pathogenic bacteria resulting in genetically modified bacteria,
30 which are attenuated bacteria.

[0043] In some embodiments, the genetically modified bacteria further include a disrupted expression of one or more bacteria virulence factor(s).

[0044] Advantageously, the system and methods described herein do not require antibiotic markers to retain the gene disruptions.

[0045] Such system and methods generated unexpected results. For example, in many cases, key bacterial virulence factors are encoded on virulence plasmids, and while the inactivation of such systems could provide an effective approach to generate genetically modified bacteria that are attenuated bacteria, a drawback to targeting plasmid-encoded genes is the potential for the regain of virulence. Indeed, such genetically modified bacteria can regain virulence through either conjugative transfer of a virulence plasmid from the natural host flora and/or the potential for genetic recombination resulting in a regain of the inactivated gene or system. The potential for reversing genetically modified live bacteria to become virulent is generally perceived as being a significant limitation of targeting plasmid-encoded virulence factor genes. Surprisingly, the inventors discovered that the system and methods described herein generated genetically modified bacteria that are attenuated and having reduced risk to regain virulence. This is demonstrated with retention of the attenuation phenotype despite several bacteria culture passages and in the absence of antibiotic markers – such advantageous properties are demonstrated even when the bacterial virulence factors are encoded on virulence plasmids.

[0046] The reader will readily understand in view of the teachings and data presented in the present application that the methods and system described herein can be used to obtain genetically modified bacteria from any virulent (pathogenic) parental bacteria containing a virulence plasmid. For example, from any virulent (pathogenic) bacteria containing a virulence plasmid present in disease-causing wild-type field strains. The system and methods described herein represent a platform technology for generating genetically modified bacteria that are attenuated bacteria.

[0047] The following paragraphs discuss bacteria characteristics that equally apply to genetically modified bacteria described herein as well as to the virulent (pathogenic) parental bacteria used for making the genetically modified bacteria.

[0048] In some embodiments, the bacteria can be Gram-negative bacteria.

[0049] In some embodiments, the bacteria can belong to the Enterobacteriaceae family, which comprises a family of gram-negative, rod-shaped, facultatively anaerobic bacteria found in soil, water, plants, and animals, which frequently occur as pathogens in vertebrates.

[0050] The term "Gram-negative bacteria" as used herein includes, but is not limited to, any one of the following bacteria: *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Anaeromyxobacter dehalogenans*, *Bordetella bronchiseptica*, *Bordetella parapertussis*, *Bordetella pertussis*, *Bradyrhizobium japonicum*,
5 *Burkholderia cenocepacia*, *Burkholderia cepacia*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Chlamydia muridarum*, *Chlamydia trachomatis*, *Chlamydomydia abortus*, *Chlamydomydia pneumoniae*, *Chromobacterium violaceum*, *Citrobacter rodentium*, *Desulfovibrio vulgaris*, *Edwardsiella tarda*, *Endozoicomonas elysicola*, *Erwinia amylovora*, *Escherichia albertii*, *Escherichia coli*, *Lawsonia intracellularis*,
10 *Mesorhizobium loti*, *Myxococcus xanthus*, *Pantoea agglomerans*, *Photobacterium damsela*, *Photobacterium luminescens*, *Photobacterium temperate*, *Pseudoalteromonas spongiae*, *Pseudomonas aeruginosa*, *Pseudomonas plecoglossicida*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Rhizobium* sp, *Salmonella enterica* and other *Salmonella* sp, *Shigella flexneri* and other *Shigella* sp, *Sodalis glossinidius*, *Vibrio*
15 *alginolyticus*, *Vibrio azureus*, *Vibrio campellii*, *Vibrio caribbenthicus*, *Vibrio harvey*, *Vibrio parahaemolyticus*, *Vibrio tasmaniensis*, *Vibrio tubiashii*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xanthomonas oryzae*, *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis*.

[0051] In some embodiments, the bacteria can be at least one of *Escherichia coli* (*E. coli*), *Shigella*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*,
20 *Serratia*, *Proteus*, *Morganella*, *Providencia* and *Yersinia*.

[0052] In some embodiments, the bacteria can be *Salmonella* or *E. coli*.

[0053] In some embodiments, the virulent (pathogenic) parental bacteria are avian pathogenic *Escherichia coli* (APEC), a subgroup of *Escherichia coli* strains that cause
25 disease in poultry. APEC infections can lead to respiratory diseases, septicemia, and other health issues in birds, significantly impacting the poultry industry.

[0054] In some embodiments, the system, methods, and genetically modified bacteria described herein offer one or more of the following technical advantages.

[0055] For example, the system and methods described herein afford means of
30 generating and retaining gene disruptions without requiring selective agents such as antibiotics. This approach provides the possibility of using the herein described genetically modified bacteria in environments where selection with chemical supplements is not feasible or desirable, such as in some animal or human

applications (such as live vaccines or live therapeutic cargo), for use in the environment, or for certain industrial processes. This approach also reduces manufacturing costs associated with antibiotic costs, waste treatment and disposal. This approach also addresses the current trend in agriculture and medical industry, which aims to reduce antibiotic use due to the potential risk of generation of increased resistance of microbes to antibiotics that are released in the environment.

[0056] For example, the genetically modified bacteria described herein lack an antibiotic selection marker capable of polluting the environment, inducing adverse effects or triggering harmful reactions in hosts, particularly during a vaccination or therapeutic cargo procedure. The methodology, system, and genetically modified bacteria described herein are designed to eliminate prospective environmentally harmful genes from the ultimate recombinant organism, such as antibiotic resistance genes.

[0057] For example, the system, methods, and the genetically modified bacteria described herein can afford a constitutive expression of at least one gene of interest introduced into the bacteria without the requirement for selective agents such as antibiotics, and can also afford the visual identification of genetically modified bacteria through inoculating the bacteria in growth media in presence of a chromogenic substrate of alkaline phosphatase.

[0058] For example, the system and methods described herein afford generating genetically modified bacteria having stable mutations in bacteria virulence factors with a reduced risk of reversion. For example, the present inventors have successfully shown mutation stability over at least 5, and at least 10 (see FIG. 19) bacteria passages. Such reduced risk of reversion to become virulent provides an opportunity to use the genetically modified bacteria in various applications, such as live vaccines, live immunogenic compositions, or live therapeutic cargo. For example, for use in animals or humans.

[0059] For example, the system, methods, and genetically modified bacteria described herein can afford live vaccines, live immunogenic compositions, or live therapeutic cargo that are of low cost to manufacture or prepare, which are safe, and which can be efficiently administered to animals or humans by practical and cost-effective methods.

System and methods

1. Chromosomal disruptions

[0060] In some embodiments, the system includes bacteria having a disruption in a chromosomal gene coding for a protein required for the synthesis of an essential component for the bacteria. In other words, when the bacteria include such disruption, the bacteria will then require an exogenous form of such essential component for growth and/or survival – i.e., the bacteria is auxotrophic for such essential component. Such bacteria is also referred to in this text as auxotrophic bacteria. Such a gene is also referred to in this text as an auxotrophic gene.

[0061] Since the system can be used in the context of a platform technology for generating several strains of genetically modified bacteria, the system can include a plurality of bacteria strains, where each bacteria strain includes a chromosomal disruption in a respective auxotrophic gene. In some embodiments, the auxotrophic gene can be the same auxotrophic gene in all bacteria strains of a given platform.

[0062] The bacteria described herein are typically derived from corresponding virulent pathogenic bacteria containing a virulence plasmid. For example, from disease-causing wild-type field bacteria strains.

[0063] In some embodiments, the herein described bacteria have a disruption in more than one chromosomal gene. For example, a disruption in an auxotrophic gene and in one or more gene(s) involved in phosphate regulation.

[0064] In some embodiments, the herein described bacteria have a disruption in a plurality of genes involved in phosphate regulation. For example, in one or more gene(s) of the phosphate transport system (*pst* operon).

[0065] In some embodiments, the auxotrophic gene and the one or more gene(s) involved in phosphate regulation are contiguous chromosomal genes.

[0066] In some embodiments, the disruption can be performed with any suitable genetic engineering technique in molecular biology by which the genome of living organisms may be modified, i.e., the chromosomal disruption may include an insertion, deletion, modification, or replacement of DNA. For example, the disruption can be performed with any genome editing techniques known in the art, such as but without being limited to techniques that involve polymerase chain reaction (PCR), homologous recombination, transposons, Clustered Regularly Interspaced Short

Palindromic Repeats (CRISPR) gene editing, Zinc-finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), and the like.

[0067] In some embodiments, the disruption is a chromosomal deletion. For example, deletion of at least a portion of the auxotrophic gene and/or of the one or more gene(s) involved in phosphate regulation.

[0068] In some embodiments, the disruption is a chromosomal deletion of the entire auxotrophic gene.

[0069] In some embodiments, the disruption is a chromosomal deletion resulting from a one-step deletion, where both the auxotrophic gene and the one or more gene(s) involved in phosphate regulation are deleted through a single genome-editing event. For example, the one-step deletion can be performed on contiguous chromosomal genes using homologous recombination.

2. Auxotrophic gene

[0070] In some embodiments, the auxotrophic gene can be any suitable auxotrophic gene. For example, a gene selected from genes involved in cell wall synthesis, in amino acid synthesis, nucleic acid synthesis, cofactor synthesis, coenzyme synthesis (e.g., FAD, NAD), etc.

[0071] In some embodiments, the auxotrophic gene is required for bacterial cell wall peptidoglycan synthesis.

[0072] In some embodiments, the auxotrophic gene is the *glmS* gene.

[0073] The *glmS* gene codes for the enzyme Glutamine-fructose-6-phosphate amidotransferase (GFAT), which catalyzes the formation of glucosamine-6-phosphate (GlcN6P), a compound essential for cell wall biosynthesis. GFAT includes enzymes that correspond to Enzyme Commission Number EC 3.5.99.6 and is required for *de novo* bacterial production of N-acetyl glucosamine (NAG).

[0074] Bacteria having a disruption in the *glmS* gene are unable to grow and/or survive, without supplementation of NAG or glucosamine (Sarvas M (1971) J Bacteriol 105: 467-471; Wu et al. J Bacteriol 105: 455-466). This is because such bacteria are unable to produce endogenous stores of NAG. Once any exogenous NAG is depleted, cell lysis and death follow, as NAG is a key building block for the bacterial cell wall

component peptidoglycan. NAG is also a component of the Lipid A core of bacterial liposaccharide.

[0075] Mutated bacteria that include a disruption in the *glmS* gene are, therefore, auxotrophic for NAG or glucosamine.

5 3. Gene(s) involved in phosphate regulation

[0076] In some embodiments, the one or more gene(s) involved in phosphate regulation includes one or more gene(s) of the phosphate transport system operon (*pst* operon).

[0077] The *pst* operon codes for a high-affinity inorganic phosphate transporter system found in many bacteria species, including both Gram-positive and Gram-negative bacteria species, e.g., in *E. coli*, *Salmonella*, *S. lividans* and *Bacillus subtilis*. The *pst* operon includes a series of genes that encode four Pst proteins, namely PstS, PstC, PstA, and PstB. In *B. subtilis* there are two copies of PstB (PstB1 and PstB2).

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[0078] The *pst* operon in *Streptomyces* and most other bacteria is organized as an ATP-binding cassette (ABC) that comprises the four proteins. The Pst system has been characterized in *E. coli* to fulfill two functions: (i) the transport of inorganic phosphate, and (ii) the negative regulation of the phosphate regulon (a complex of 20 proteins mostly related to organic phosphate transport). It is believed that these two functions are not related, since the repressibility of the phosphate regulon depends on the integral structure of the Pst system and not on the inorganic phosphate transported. Another gene of the *pst* operon, *phoU*, produces a protein involved in the negative regulation of the Pho regulon.

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[0079] In some embodiments, the herein described disruption in the *pst* operon can include a disruption in one or more gene(s) of the *pst* operon, preferably in a plurality of genes of the *pst* operon. For example, the herein described disruption in the *pst* operon includes a disruption in one or more gene(s) selected from *pstS*, *pstC*, *pstA*, and *pstB*, preferably in one or more gene(s) selected from *pstS*, *pstC*, and *pstA*, and more preferably in the *pstS*, *pstC*, and *pstA* genes.

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[0080] Disruption of the *pst* operon results in deregulation of the phosphate regulon, and constitutive expression of genes under the control of PhoB-regulated promoters. This constitutive expression capability provides a technical effect in that the genetically modified bacteria described herein will constitutively express any gene

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(i.e., endogenous or recombinant) which is under the control of a PhoB-regulated promoter, since this genetically modified bacteria has a disruption in the *pst* operon. This can allow overexpressing an endogenous gene or constitutively expressing an exogenous (recombinant) gene of interest, when such gene is under the control of a
5 PhoB-regulated promoter.

[0081] In some embodiments, the genetically modified bacteria described herein further includes a recombinant gene of interest to deliver a therapeutic payload. For example, the recombinant gene of interest can be present in the rescue DNA molecule, as will be described later in this text.

10 [0082] In some embodiments, the therapeutic payload includes a therapeutic gene or an antigen-coding gene.

[0083] In some embodiments, the recombinant gene of interest to deliver a therapeutic payload is under the control of a PhoB-regulated promoter. This recombinant gene of interest can be constitutively expressed when the genetically
15 modified bacteria has a disrupted *pst* operon.

[0084] In some embodiments, the genetically modified bacteria described herein constitutively expresses the *phoA* gene, which codes for alkaline phosphatase. This is because the *phoA* gene is under the control of a PhoB-regulated promoter. Disruption of the *pst* operon thus results in constitutive expression of PhoA.
20 Advantageously, the constitutive expression of PhoA allows for direct identification of the genetically modified bacteria under specific conditions. For example, the specific conditions may include growing the bacteria in growth media supplemented with a chromogenic substrate for alkaline phosphatase. For example, the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate di-sodium (BCIP), alone or together
25 with Nitroblue tetrazolium (NBT), Fast Red-naphthol, 6-chloro-3-indolyl-phosphate p-toluidine salt (salmon phosphate), or 5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt (magenta phosphate). Growth of bacteria on growth media (e.g., growth media plates) supplemented with a chromogenic substrate for alkaline phosphatase results in the generation of colored colonies (e.g., dark blue when using BCIP), thus
30 providing a direct visual identification process.

[0085] In some embodiments, disruption of the *pst* operon affords a method for identification of bacteria having the herein described disruption. This is because in bacteria where the *pst* operon is inactivated/deleted, the *pho* regulon is constitutively

expressed. As such, even under high phosphate conditions, the bacteria will highly express the alkaline phosphatase enzyme, which can provide a means of detecting the bacteria. The method may include obtaining a sample suspected of containing the bacteria having the disruption in the *pst* operon and inoculating the sample in growth media (e.g., a plate or a liquid broth) supplemented with a chromogenic substrate for alkaline phosphatase to obtain an inoculated growth media. The method then includes incubating the inoculated growth media under bacteria growth conditions, and identifying presence of bacteria having the disruption in the *pst* operon through presence of signal from the chromogenic substrate. When growing on media plate, this identification can further include identifying single colonies.

[0086] Without being bound by any theory, the present inventors are not aware of any public literature that describes the disruption of one or more *pst* genes to provide a simple differential phenotype to identify genetically modified bacteria and/or as a means of generating strains having constitutive expression of genes under the control of PhoB-regulated promoters.

4. DNA rescue molecule

[0087] In some embodiments, the system further includes a DNA rescue molecule containing a functional copy of the gene coding for the protein required for synthesizing the essential component (i.e., a functional copy of the auxotrophic gene). The herein-described mutated bacteria, when harboring the DNA rescue molecule (i.e., as recombinant bacteria), grows and/or survives when cultured in the absence of the essential component.

[0088] In some embodiments, the DNA rescue molecule is a non-chromosomal genetic element.

[0089] Advantageously, the DNA rescue molecule can be configured to cause selective retention thereof due to the functional auxotrophic gene being contained therein, which favors the selective retention of the DNA rescue molecule by the mutated bacteria when grown in media that has not been supplemented with the essential auxotrophic element, including *in vivo*. As such, retention of this DNA rescue molecule is obtained without use of antibiotic-resistance genes.

[0090] The DNA rescue molecule containing a functional copy of the auxotrophic gene can be introduced in bacteria with any suitable technique known in the art. For example, with electroporation, transformation, transduction, or conjugation. These

techniques enable the introduction of the DNA rescue molecule into the bacteria, allowing the functional gene to complement the disrupted auxotrophic gene. Suitable techniques are known in the art and will not be further described here.

[0091] In some embodiments, the DNA rescue molecule can include a site that allows the insertion of one or more recombinant gene of interest. For example, the gene of interest may be used to deliver a therapeutic payload to a host.

[0092] For example, the DNA rescue molecule can include a multiple cloning site (MCS) or polylinker having restriction enzyme sites to clone DNA molecules therein. For example, the one or more recombinant gene of interest can include a therapeutic gene, an antigen-coding gene, a biosensor, an enzyme, an antibody, and the like. For example, the gene of interest can be operably linked to and controlled by a PhoB-regulated, i.e., for constitutive expression in the genetically modified bacteria described herein.

[0093] For example, the DNA rescue molecule can encode a protein of interest that provides a therapeutic benefit to the host. This therapeutic benefit may arise from the protein's ability to modulate a physiological process, correct a genetic defect, or combat a pathogenic condition within the host. In such embodiments, the live genetically modified bacteria function as a live therapeutic cargo, delivering the encoded protein directly to the host's cells or tissues in a targeted and sustained manner. The therapeutic protein may include enzymes, cytokines, hormones, or other biologically active molecules designed to address specific medical conditions. Furthermore, the genetically modified bacteria can be engineered to express the therapeutic protein in response to environmental or host-specific cues, enhancing precision and minimizing off-target effects. This approach can enable the bacteria to serve as a dynamic delivery system, offering a novel platform for the treatment of various diseases, including genetic disorders, metabolic conditions, and infectious diseases.

[0094] In some embodiments, the bacteria can deliver the encoded protein in a targeted manner through several engineered mechanisms or natural behaviors, such as tissue tropism (e.g., natural affinity for specific tissues or cells, such as preferential colonization of the gut, lungs, or tumors, allowing targeted delivery of therapeutic proteins to these regions, etc.), surface display of targeting molecules (e.g., expression of ligands, peptides, or antibodies on the bacteria surface, which bind selectively to receptors on target cells, etc.), environmental triggers (e.g., bacteria can

be programmed to activate the expression and release of the therapeutic protein in response to specific environmental conditions, such as pH changes, hypoxia, or the presence of certain metabolites, etc.), genetic switches and inducible systems (e.g., bacteria can be equipped with genetic circuits that restrict protein expression to particular conditions or tissues, etc.), encapsulation of formulation (e.g., bacteria can be encapsulated in delivery systems, such as hydrogels or nanoparticles, that direct them to specific tissues, etc.), and the like.

[0095] In some embodiments, the DNA rescue molecule is configured to disrupt expression of one or more bacteria virulence factor(s).

[0096] For example, the DNA rescue molecule can be configured to disrupt one or more bacteria gene(s) involved in one or more virulence pathway(s). For example, the one or more bacteria gene(s) involved in one or more virulence pathway(s) can code for, but without being limited to, transcription factor(s) (i.e., sequence-specific DNA-binding proteins required to modulate gene expression) regulating expression of corresponding bacteria virulence factor(s), RNA binding protein(s) regulating stability and/or degradation of messenger RNA coding for corresponding bacteria virulence factor(s) (e.g., the Hfq protein (also known as HF-I protein) encoded by the *hfq* gene), and the like.

[0097] For example, the DNA rescue molecule can be configured to disrupt one or more bacteria virulence gene(s) coding for respective one or more bacteria virulence factor(s). Non-limiting examples of bacteria virulence gene(s) will be discussed later in this text.

[0098] In some embodiments, the DNA rescue molecule can take the form of a plasmid vector. For example, the plasmid vector may be a mini-plasmid, which is typically a small and simplified plasmid designed to reduce unnecessary sequences and enhance efficiency, or a traditional plasmid, which includes more extensive regulatory elements and features. These plasmid vectors may carry additional elements such as promoters, enhancers, or terminators to facilitate gene expression and selection in the host organism.

[0099] For example, when the DNA rescue molecule takes the form of a plasmid vector, the DNA rescue molecule can be configured to cause selective loss of a bacteria virulence plasmid containing the one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s). For example, the selective loss mechanism

of the bacteria virulence plasmid containing the one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s) can be implemented through the well-known plasmid incompatibility mechanism. In other words, the DNA rescue molecule can contain an origin of replication which is incompatible with that one of the bacteria virulence plasmid contained in the virulent pathogenic bacteria, thus causing the selective loss of the virulence plasmid. This selective loss is non-dependent on antibiotic markers since the DNA rescue molecule contains a functional copy of the auxotrophic gene. Such selective loss of the bacteria virulence plasmid containing the one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s) generates the genetically modified bacteria described herein.

[0100] In the non-limiting specific implementation of APEC, *Salmonella* and many other enterobacteria, most virulence plasmids are of single-copy (1 per cell) and containing replication origins similar to the F-plasmids. Other plasmids with low copy can also be eliminated over time by replacement with a recombinant plasmid that selects for survival under certain growth conditions (i.e., when a strain is an auxotroph for an essential compound required for bacterial viability). When a rescue plasmid encoding *glmS* is introduced, the original plasmid is lost when cells are grown in the absence of NAG as both plasmids have the same incompatibility group and the *glmS* encoding plasmid is selected for as it allows growth of the bacterial cells in the absence of NAG.

[0101] In some embodiments, the DNA rescue molecule in the form of a plasmid can be based on a bacteria virulence plasmid. In such instances, the bacteria virulence plasmid can be modified to disrupt the one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s). As discussed elsewhere, this disruption in the one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s) may include a mutation, insertion, or deletion.

[0102] In some embodiments, the DNA rescue molecule can take the form of a DNA cassette.

[0103] For example, when the DNA rescue molecule takes the form of a DNA cassette, the DNA rescue molecule can be configured to cause selective loss of one or more bacteria virulence gene(s) coding for the bacteria virulence factor(s). For example, through insertion of the DNA cassette into the one or more bacteria gene involved in one or more virulence pathway(s) and/or into the one or more bacteria virulence gene(s) coding for one or more bacteria virulence factor(s). This can be

done by basic allelic exchange using methods such as *sacB*-based suicide vectors (e.g., pMEG). Double cross-over exchange results in introduction of the auxotrophic gene present on the DNA rescue molecule into the one or more virulence gene(s) resulting in the herein described disruption.

- 5 [0104] In some embodiments, the plasmid vector is a copy of the bacteria virulence plasmid that has been modified with insertion of a functional copy of the auxotrophic gene in a DNA location corresponding to the one or more genes virulence gene(s) present in the bacteria virulence plasmid.

10 5. Bacteria virulence gene(s)

[0105] In some embodiments, the genetically modified bacteria includes a disruption in one or more bacteria virulence gene(s) coding for one or more bacteria virulence factor(s) normally present in the virulent pathogenic bacteria.

- 15 [0106] For example, the one or more bacteria virulence gene(s) can be one or more gene(s) from the siderophore system. Siderophores are low-molecular-weight (500–1,500 Da) high-affinity iron-chelating compounds for solubilization and transport of ferric iron into bacterial cells. In the extracellular milieu, secreted siderophores form soluble iron–siderophore complexes with ferric iron. The soluble iron–siderophore complexes are actively transported into bacterial cells via specific outer membrane
20 receptors, then ferric iron is released and reduced to ferrous iron, which can be used for cellular needs. Siderophores are not only essential for the growth of most pathogenic bacteria but also play important roles in non-iron metal transport, protection against oxidative stress, antibiotic activity, interspecies interactions, and virulence (e.g., Kramer et al., (2020). Bacterial siderophores in community and host
25 interactions. *Nat. Rev. Microbiol.* 18, 152–163).

[0107] In some embodiments, the one or more bacteria virulence factor(s) are coded by one or more gene(s) from the *iuc* operon and/or the *iro* operon.

- 30 [0108] The *iuc* operon includes the *iucABCD* genes. The *iucABCD* genes are required to synthesize the iron-chelating siderophore aerobactin. Aerobactin, a citrate-hydroxamate siderophore, is produced by many pathogenic bacteria. The aerobactin operon encodes four biosynthetic enzymes (*iucABCD*) and a transmembrane transporter (*iutA*) involved in aerobactin siderophore biosynthesis and transport.

Aerobactin is known to contribute to bacterial virulence in many bacteria (including *Escherichia*, *Vibrio*, *Salmonella*, and *Shigella*) by mediating iron acquisition during infection (Sheldon et al. (2016). Iron acquisition strategies of bacterial pathogens. *Microbiol. Spectr.* 4(2)).

- 5 [0109] The *iro* operon includes the *iroBCDEN* genes. The *iroBCDEN* genes encode the salmochelin siderophore system present in *Salmonella enterica* and some extraintestinal pathogenic *E. coli* (ExPEC) strains. Avian pathogenic *Escherichia coli* (APEC) strains are a subset of ExPEC strains associated with respiratory infections and septicemia in avian species, in particular poultry. The iron-chelating siderophore
10 salmochelin is known to contribute to bacterial virulence by mediating iron acquisition during infection (e.g. Caza et al., Infect Immun. 2008 Aug; 76(8): 3539–3549).

[0110] In some embodiments, the DNA rescue molecule can be configured to disrupt expression of the *iuc* operon, such as to disrupt one or more genes from the *iucABCD* genes.

- 15 [0111] For example, the DNA rescue molecule in the form of a plasmid vector can be configured to cause selective loss of a bacteria virulence plasmid containing a functional *iuc* operon. The DNA rescue molecule can be further configured to be devoid of a functional copy of the *iuc* operon, or can be free of one or more genes from the *iucABCD* genes.

- 20 [0112] For example, the DNA rescue molecule in the form of a cassette can be configured to insert into the *iuc* operon, i.e., to disrupt one or more genes from the *iucABCD* genes through insertion therein.

- [0113] In some embodiments, the DNA rescue molecule can be configured to disrupt expression of the *iro* operon, such as to disrupt one or more genes from the
25 *iroBCDEN* genes.

- [0114] For example, the DNA rescue molecule in the form of a plasmid vector can be configured to cause selective loss of a bacteria virulence plasmid containing a functional *iro* operon. The plasmid vector can be further configured to be devoid of a functional copy of the *iro* operon, or can be free of one or more genes from the
30 *iroBCDEN* genes.

[0115] For example, the DNA rescue molecule in the form of a cassette can be configured to insert into the *iro* operon, i.e., to disrupt one or more genes from the *iroBCDEN* genes through insertion therein.

5 [0116] For example, the DNA rescue molecule in the form of a plasmid vector can be configured to cause selective loss of a bacteria virulence plasmid containing a functional *iuc* operon and a functional *iro* operon. The DNA rescue molecule can be further configured to be devoid of functional copies of the *iuc* operon and the *iro* operon. The resulting genetically modified bacteria will have an even greater reduction in its virulence compared to genetically modified bacteria with a single disruption in
10 the siderophore system.

[0117] In some embodiments, the plasmid vector is a copy of the bacteria virulence plasmid that has been modified with insertion of a functional copy of the auxotrophic gene in a DNA location corresponding to one or more genes of the siderophore system in the bacteria virulence plasmid.

15 [0118] In some embodiments, the DNA rescue molecule in the form of a cassette may be configured to disrupt both *iuc* and *iro* operons. The resulting bacteria will also have an even greater reduction in its virulence compared to bacteria with a single disruption in the siderophore system.

[0119] In some embodiments, the herein described system and methods disrupt
20 expression of two pathogen-associated siderophore synthesis systems, namely genes for the salmochelin and the aerobactin siderophores. Such disruption can occur through deletion or mutation of aerobactin synthesis operon genes (*iucABCD*), and through deletion or mutation of salmochelin synthesis and utilization genes (*iroBCDE*). In such embodiments, the modifications to inactivate the patho-specific siderophore
25 synthesis systems (encoding salmochelin and aerobactin) renders the bacteria strains unable to persist in extra-intestinal tissues of the host.

[0120] Since the siderophore outer-membrane receptors *lutA* required for aerobactin uptake and *IroN* required for salmochelin uptake are upregulated during infection in the respiratory tract and bloodstream and are surface-expressed patho-specific
30 antigens, the genes encoding the receptors were retained in the system and methods described herein. The genetically modified bacteria described herein, in particular the genetically modified *E. coli* described herein strongly expresses these genes under low-iron conditions.

[0121] Importantly, the genetically modified strains described herein are still able to grow well *in vitro* under low iron conditions as they produce and can uptake the siderophore enterobactin. For example, this is the only siderophore present in many strains of *E. coli* residing as commensals in the mammalian or avian intestine.

5 Although enterobactin is an excellent siderophore *in vitro* or in environmental niches such as soil, it is less effective for iron uptake during host infection as host defense proteins such as lipocalin 2 (siderocalin) sequester this siderophore, making it unavailable for iron uptake by the bacteria in the host.

[0122] The capture of enterobactin by lipocalin 2 (siderocalin) has also been shown
10 to elicit a host inflammatory response and it's production may serve as a signal to the host immune system (Nelson AL, Ratner AJ, Barasch J, Weiser JN. Interleukin-8 secretion in response to aferric enterobactin is potentiated by siderocalin. Infect Immun. 2007 Jun; 75(6):3160-8. doi: 10.1128/IAI.01719-06. Epub 2007 Apr 9. PMID: 17420239).

15 [0123] Maintaining the enterobactin siderophore system can, therefore, provide some bacterial growth in some tissues, such as the intestine, but a very limited capacity to sequester iron in extra-intestinal tissues or respiratory mucosal surfaces, where the host defense lipocalin 2 can neutralize this siderophore and hence impede bacteria growth.

20 6. Immunogenic compositions – vaccines

[0124] In some embodiments, the genetically modified bacteria can be administered (e.g., in the form of an immunogenic composition or vaccine) to a host with the ability to elicit, induce, or stimulate an immune response. Such host can encompass a wide range of vertebrate hosts, such as mammals (including domestic, agricultural,
25 laboratory animals, and humans), various avian species (including domestic and agriculturally significant birds, such as chickens, ducks, turkeys, geese, bantams, quail, pheasant, pigeons, and the like), and various fish species.

[0125] In some embodiments, the vaccine disclosed herein comprises live, non-virulent genetically modified bacteria that are particularly advantageous for
30 vaccinating vertebrate hosts susceptible to diseases caused by corresponding virulent (pathogenic) bacteria. The genetically modified bacteria used in the vaccine are auxotrophic, possessing a non-reverting, non-leaky mutation in a biosynthetic pathway. This mutation results in a requirement for an essential component that is not

available in sufficient quantities within the animal to be vaccinated to allow significant proliferation of the genetically modified bacteria. Accordingly, the vaccine strains can be cultured on media supplemented with the essential component and, upon introduction into the host, will remain viable until eliminated by the host's immune response. However, these bacteria will be unable to undergo significant multiplication due to the unavailability of the biosynthetic pathway product in the host. Despite this limitation, the genetically modified bacteria retain their native antigenic characteristics, enabling them to stimulate an effective immune response.

[0126] In addition to the auxotrophic mutation that restricts bacterial multiplication within the vaccinated host, the genetically modified bacteria used as live vaccines include a genetic marker to facilitate differentiation from other bacteria of the same species, whether wild-type strains or other live vaccine strains. A suitable genetic marker includes, for example, the constitutive expression of the *phoA* gene which affords identification with a chromophore, such as BCIP. Such markers are particularly beneficial in distinguishing the vaccine strain from wild-type strains, especially in cases where a vaccinated subject develops a bacterial infection due to exposure prior to the establishment of vaccine-induced immunity.

[0127] Unlike other live vaccine formulations, the present invention explicitly identifies the mechanism responsible for the loss of virulence in the genetically modified bacteria. In contrast to other live vaccine strains that are rendered non-virulent through alterations in lipopolysaccharide structure, the vaccine strains described herein retain substantially unaltered O-antigenic characteristics and other surface antigens associated with virulence and immunity, such as major outer membrane proteins. Consequently, these vaccines are capable of inducing the production of anti-O antibodies, which are recognized as critical components of immunity elicited by vaccination. The vaccine strains are expected to persist within the host for extended durations, typically weeks, thereby enhancing the immunizing effect through continuous stimulation of the host immune system until the immune system has eradicated all bacterial organisms.

[0128] In some embodiments, the genetically modified bacteria, an immunogenic composition or vaccine containing the same, can be administered for prophylactic or therapeutic purposes.

[0129] In some embodiments, the genetically modified bacteria is alive when administered to the host in an immunogenic composition or vaccine. The live

genetically modified bacteria can thus be formulated into a live immunogenic composition or live vaccine.

[0130] In some embodiments, the immunogenic composition or vaccine containing the genetically modified bacteria is capable of eliciting a protective immune response in an animal against subsequent natural exposure to, or experimental challenge with, the corresponding virulent pathogenic bacteria.

[0131] Methods of administering the immunological composition or vaccine described herein encompass well-known techniques within the expertise of those with ordinary skills in the field.

[0132] For example, the immunogenic composition or vaccine, as described herein, can be formulated for parenteral or enteral administration.

[0133] For example, the immunogenic composition or vaccine as described herein can be for a suitable administration such as oral, ocular, transdermal, transmucosal, intravenous, subcutaneous, intramuscular, intradermal, intraperitoneal, intracapsular, intraspinal, intrasternal, intrapulmonary, intranasal, vaginal, rectal, intraocular, intrathecal, buccal (e.g., sublingual), respiratory, topical, ingestion, local delivery (e.g., by aerosol or transdermal), and similar approaches.

[0134] For example, the immunogenic composition or vaccine, as described herein, can be for a suitable administration, such as with ocular (eye drops), pulmonary inhalation, nasal delivery, or respiratory delivery. By "nasal delivery", it is intended that the immunological composition or vaccine described herein is administered to the subject through the nose. By "pulmonary inhalation", it is intended that the immunological composition or vaccine described herein is administered to the subject through the airways in the nose or mouth so as to result in the delivery of the immunological composition or vaccine described herein to the lung tissues and into the interior of the lung. Both nasal delivery and pulmonary inhalation can result in the delivery of the immunological composition or vaccine described herein to the lung tissues and into the interior of the lung, also referred to herein as "pulmonary delivery." By "respiratory delivery", it is intended that the immunological composition or vaccine described herein is administered to the subject through the respiratory system of the subject so as to result in the delivery of the immunological composition or vaccine described herein to the organs and tissues of the respiratory system of the subject

organism. The organs and tissues of the respiratory system of a subject organism include, but are not limited to, the lungs, or nose.

[0135] In some embodiments, the immunogenic composition or vaccine, as described herein, can take the form of a ready-to-use solution for parenteral injection.

- 5 [0136] In some embodiments, the immunogenic composition or vaccine, as described herein, can take the form of a lyophilized, frozen, spray dried, or foam-dried form composition. For example, the immunogenic composition or vaccine can take the form of a powder.

- 10 [0137] In some embodiments, the immunogenic composition or vaccine, as described herein, can take the form of a composition intended for reconstitution in a physiologically acceptable solution for administration through any suitable route.

- 15 [0138] In some embodiments, a commercial package may include in separate containers the composition and a physiologically acceptable solution, for reconstitution in a physiologically acceptable solution for administration through any suitable route. Such a commercial package may also further include instructions for using, mixing, or handling the composition and the physiologically acceptable solution. Such a commercial package may also further include a dispenser adapted for the administration of the reconstituted composition to an animal.

- 20 [0139] In some embodiments, a physiologically acceptable solution may include one or more elements suitable for reconstitution of the composition for administration through any suitable route. For example, sterile water for injection is water that has been purified and sterilized to meet the standards for injectable use. For example, normal saline (0.9% sodium chloride) is a solution that contains a concentration of sodium chloride similar to that of the body's extracellular fluid.

- 25 [0140] In some embodiments, the immunogenic composition or vaccine may further comprise a pharmaceutical or veterinary-acceptable excipient, diluent, or carrier.

[0141] Pharmaceutical or veterinary-acceptable excipients, diluents, or carriers are well known to the one skilled in the art. Examples include a 0.9% NaCl solution (saline) and buffers used to maintain a proper pH (e.g., Phosphate, HEPES, etc.).

- 30 [0142] Details on doses and volumes are provided in the general description and can be determined by skilled artisans through careful consideration of this disclosure alongside existing knowledge in the field, without undue experimentation.

[0143] The dosages required will vary with the antigenicity of the bacteria (or antigen expressed therefrom) and need only be an amount sufficient to induce an immune response sufficient to confer protection against the target pathogenic organism. Routine experimentation will easily establish the required amount.

- 5 [0144] For example, an initial vaccine dosage for chicken having 40-50g body weight at the time of administration could be of 5×10^6 CFU to 5×10^8 CFU per chick (CFU = colony forming unit). For sake of clarity, a dosage of about $1-2 \times 10^9$ CFU/Kg would correspond to a dosage of 1×10^8 CFU for chicken having 40-50g body weight.

7. Therapeutic cargo systems

- 10 [0145] In some embodiments, the genetically modified bacteria described herein can be utilized as a therapeutic cargo system, wherein the bacteria are engineered to deliver therapeutic agents to a host cell, organ or tissue – so called “bactofection”. These therapeutic agents can include proteins, peptides, nucleic acids, small molecules, or other biologically active substances that confer a therapeutic benefit.
- 15 The bacteria serve as a live delivery platform, capable of transporting these agents to specific tissues or cells within the host in a targeted and sustained manner.

- [0146] The genetically modified bacteria can be engineered to carry DNA rescue molecules encoding proteins of interest that address specific medical conditions. For example, such proteins may include enzymes to correct metabolic disorders,
- 20 cytokines to modulate immune responses, hormones to restore physiological balance, and the like. These proteins can be expressed by the bacteria and released directly at the site of action, providing localized therapeutic effects while minimizing systemic side effects.

- [0147] In some embodiments, the genetically modified bacteria can target solid
- 25 tumors, leading to therapeutic effects in a tumor-bearing subject. In such cases, an anaerobic bacteria is preferably employed. Without being bound by any theory it is believed that various micro-environment characteristics might favor the preferential proliferation and colonization of bacteria in tumors. For example, the hypoxic micro-environment present in necrotic areas of solid tumors can drive the genetically
- 30 modified bacteria to target solid tumors. For example, chemo-attracting compounds present in necrotic regions (e.g., aspartate, serine, citrate, ribose or galactose) produced by quiescent cancer cells can be important contributing factors for bacterial chemotaxis towards tumors. For example, other unique micro-environment properties

found within solid tumors such as aberrant neo-vasculature and localized immunosuppression are also believed to be factors involved in bacterial tumor-targeting.

5 [0148] In some embodiments, the genetically modified bacteria are engineered for tissue-specific targeting. This may involve the expression of ligands or surface molecules that bind selectively to receptors present on the target tissue or cell type. For instance, bacteria can be designed to preferentially colonize tumors, inflamed tissues, or other diseased areas, ensuring precise delivery of the therapeutic cargo.

10 [0149] In some embodiments, the therapeutic delivery can be controlled by environmental or host-specific cues. For example, bacterial genetic circuits can be engineered to activate the expression or release of the therapeutic agent in response to environmental conditions such as pH, hypoxia, or specific metabolites. This ensures that the therapeutic cargo is deployed only under appropriate conditions, enhancing safety and efficacy.

15 [0150] The genetically modified bacteria can also employ secretion systems such as type III, type IV, or type VI secretion systems to deliver therapeutic proteins directly into the cytoplasm of host cells. This allows for efficient intracellular delivery of therapeutic agents, which is particularly beneficial for conditions requiring intracellular modulation, such as genetic disorders or infections.

20 [0151] In some embodiments, the bacteria can be administered in a variety of forms, including as part of a pharmaceutical composition. The composition may include a physiologically acceptable carrier, excipient, or diluent suitable for the route of administration, such as oral, intravenous, intranasal, or subcutaneous. For example, the bacteria may be encapsulated in a biocompatible matrix or nanoparticle
25 formulation that enhances stability and targeting while protecting the bacteria during transit to the target site.

[0152] The dosage and administration schedule for the therapeutic bacteria will depend on various factors, including the type of therapeutic agent, the condition being treated, and the specific requirements of the host. In general, the dosage should be
30 sufficient to deliver an effective amount of the therapeutic agent to achieve the desired therapeutic outcome.

[0153] The genetically modified bacteria as therapeutic cargo systems described herein present an approach to treating a wide range of diseases, including but not

limited to genetic disorders, metabolic conditions, cancer, and infectious diseases. By leveraging the inherent adaptability and targeting capabilities of bacteria, these systems offer a versatile and innovative platform for therapeutics.

8. Genetically modified bacteria

5 [0154] In a broad aspect, the present inventors have developed genetically modified bacteria which present an attenuation phenotype, which retains the genetic modification and thus the attenuation phenotype without the need of antibiotic markers.

10 [0155] In some embodiments, the genetically modified bacteria include a chromosomal disruption in an auxotrophic gene and in one or more gene(s) involved in phosphate regulation, such as one or more gene(s) of the phosphate transporter system (*pst* operon). The genetically modified bacteria further includes a DNA rescue molecule comprising a functional copy of the auxotrophic gene. The genetically modified bacteria has lost the ability to cause serious illness but retains the ability to
15 stimulate immunity, for example, against the virulent pathogenic bacteria strain corresponding to the genetically modified bacteria strain.

[0156] The genetically modified bacteria can further include a disruption of one or more bacteria virulence factor(s).

20 [0157] Specific implementations of the genetically modified bacteria and the system for making same will now be further explained with reference to FIG. 1A and 1B.

[0158] As shown in FIG. 1A and FIG. 1B, a disruption **150** in the chromosomal phosphate transporter system (*pst* operon) **100** and in the *glmS* gene **200** results in a mutated chromosome **110** having disrupted *Pst* and *glmS* genes. The disruption **150** can be performed with a one-step deletion in contiguous chromosomal genes **100**,
25 **200**, as shown. Advantageously, the disrupted *glmS* gene **200** causes a conditionally lethal mutation, and bacteria cells are unable to grow and/or survive without supplementation with NAG. Advantageously, the disrupted *pst* operon **100** causes deregulation of the Pho regulon, resulting in constitutive production of genes under the control of PhoB-dependent promoter.

30 [0159] As shown in FIG. 1B, the DNA rescue molecule containing a functional copy of the *glmS* gene can take the form of plasmid vector **250** or can take the form of DNA cassette **350**. Both the plasmid vector **250** and cassette **350** are shown as having a

functional copy of the auxotrophic *glmS* gene to complement the disrupted chromosomal *glmS* gene.

[0160] In some embodiments, the *glmS* gene on the plasmid vector **250** or cassette **350** can be under the control of a PhoB-dependent or of the promoter native to the *glmS* gene.

[0161] In some embodiments, the plasmid vector **250** or the cassette **350** can include a site that allows the insertion of exogenous DNA genes. As shown in FIG. 1B, this site can include a multiple cloning site (MCS). Examples of exogenous DNA genes are discussed elsewhere in this text.

9. Practical implementation for genetically modified *E. coli*

[0162] In some embodiments, a pathogenic *E. coli* bacteria strain can be modified as per the herein described teachings to obtain an genetically modified *E. coli* bacteria strain. For example, the genetically modified *E. coli* can be used as an immunogenic composition, therapeutic cargo or vaccine. For example, the immunogenic composition or vaccine can be used against the above pathogenic *E. coli* bacteria strain.

[0163] In some embodiments, the genetically modified bacteria described herein, immunological compositions and vaccines containing same can be useful in the context of vaccinating animals.

[0164] For example, in the context of vaccinating domestic and agriculturally significant birds such as chickens, turkeys, quails, ducks, and the like.

[0165] For example, the avian species can be vaccinated with the herein described genetically modified bacteria to target avian pathogenic *Escherichia coli* (APEC) infections and associated diseases.

[0166] APEC-associated diseases represent a significant impact on the poultry industry worldwide (e.g., chickens, turkey). Indeed, APEC are typically inadvertently maintained in the poultry house environment through fecal contamination such that systemic infections can occur when large numbers of APEC gain access to the bloodstream via the respiratory tract or intestine. Typically, bacteremia progresses to septicemia and death, or the infection can extend to serosal surfaces, pericardium, joints, and other organs. In birds, APEC generally cause an inflammation of the air

sacs (airsacculitis), an inflammation of the oviduct (salpingitis), and an inflammation of the skin and muscles (cellulitis), which all lead to tissue damage (i.e., lesions).

[0167] Typically, APEC and associated diseases can cause significant economic losses in the poultry industry via, for example, mortality, failure to gain weight (also referred to as poor feed conversion), hatchability, and/or lay, an important cause of Septicemia-toxemia (sep-tox, bacteria circulating in blood), and cellulitis, in condemnation in the broiler processing plant, or any combinations thereof.

[0168] Generally, treatment of APEC and associated diseases (e.g., avian colibacillosis) in the poultry industry entails antibiotic administration in the feed or water. However, in recent years, there have been reports of a high frequency of resistance to antibiotics such as tetracycline, kanamycin, neomycin, cephalotin, streptomycin, and erythromycin. Many APEC strains are also resistant to several antibiotics. Wide-spread sensitivity to ampicillin and chloramphenicol has also been observed. In 2012 and 2013, the United States Food and Drug Administration published "Guidance for Industry #209 and #213" advising the food-producing animal industry to use antibiotics judiciously. These documents discourage the use of antimicrobial growth promoters as a part of a set of strategies to combat antimicrobial resistance. In response to consumer demand, regulatory requirements, and scientific concerns, the US broiler industry has shifted most production to No Antibiotics Ever (NAE). The no antibiotics ever system prohibits all medically important antibiotics from being used as antimicrobial growth promoters. For example, as of 2019, over 50% of birds produced in the US are under NAE. Broilers in NAE environment are more susceptible to colibacillosis and other infections due to an increase in physiological stressors and lack of subtherapeutic antimicrobials.

[0169] In some embodiments, a pathogenic *E. coli* bacteria strain can be modified as per the herein described teachings to obtain an genetically modified *E. coli* bacteria strain, which can be used as an immunogenic composition, therapeutic cargo or vaccine. For example, the immunogenic composition or vaccine can be used against a pathogenic *E. coli* bacteria strain.

[0170] In some embodiments, the herein described DNA rescue molecule can be a plasmid vector based on the pAPEC-1 virulence plasmid (Mellata et al., PLoS One. 2009;4(1):e4232). For example, the DNA rescue molecule can be configured to disrupt expression of one or more bacteria virulence gene(s) through plasmid incompatibility with a bacteria virulence plasmid, such as the bacteria pAPEC-1

virulence plasmid. For example, the DNA rescue molecule plasmid vector based on the pAPEC-1 virulence plasmid can be configured to include a functional auxotrophic gene (such as the *glmS* gene) inserted in one or more one or more genes in the *iuc* and/or the *iro* operon, thus causing retention of the DNA rescue molecule plasmid vector over the pAPEC-1 virulence plasmid through complementation with the
5 disrupted chromosomal *glmS* gene.

[0171] In some embodiments, the genetically modified bacteria described herein can be an genetically modified APEC strain of any serotype. A non-limiting example of an genetically modified APEC strain of serotype O7 is as illustrated in FIG. 7B. This
10 bacterium is shown as still expressing O antigens, fimbriae, LPS, and the like, which are common antigens recognized by the immune system in response to infection. FIG. 7B also shows the disruption in the chromosomal *glmS* and *pst* operon, as well as the DNA rescue molecule in the form of a plasmid vector based on the virulence pAPEC-1 plasmid. The DNA rescue molecule is shown as containing a functional copy of the
15 *glmS* gene and no functional copy of the *iuc* operon and the *iro* operon.

[0172] A non-limiting example of a genetically modified *E. coli* strain (APEC) of serotype O7 is the strain deposited at the International Depository Authority of Canada (IDAC) under accession number 201223-01, which was deposited on December 21, 2023.

20 [0173] As shown in the non-limiting examples, the genetically modified O7 APEC deposited under accession number 201223-01 affords a protective immunity in an avian species against a pathogenic O7 APEC strain.

10. Practical implementation for genetically modified *Salmonella*

[0174] Salmonellosis is a worldwide occurring disease caused by bacteria belonging
25 to the genus *Salmonella*. *Salmonella enterica*, subspecies *enterica*, are Gram-negative bacterial pathogens. Known *Salmonella* pathogens include without being limited to *S. kentucky*, *S. enteritidis*, *S. heidelberg*, *S. typhimurium* and *S. hadar*.

[0175] *Salmonella enterica* serovars *enteritidis* (*S. enteritidis*; SE) and *typhimurium* are significant public health concerns associated with human consumption of
30 contaminated poultry meat and eggs. Chickens infected with these serovars are typically asymptomatic but shed the bacteria for extended periods, posing a risk to human health. Eggs are a primary vehicle for *Salmonella* transmission, with contamination arising externally from environmental exposure or internally through

reproductive tract colonization by *S. enteritidis*. Internal contamination, which hygienic measures cannot eliminate, is more likely to occur during egg formation rather than through shell penetration.

[0176] Regulatory efforts in the European Union have reduced *Salmonella* prevalence in poultry and eggs through vaccination and monitoring programs, including bans on antimicrobial use and requirements for vaccination in high-prevalence areas. Despite these measures, *Salmonella* remains a commonly reported zoonotic disease, with eggs still posing a significant risk of transmission.

[0177] In some embodiments, a pathogenic *Salmonella* bacteria strain can be modified as per the herein described teachings to obtain a genetically modified *Salmonella* bacteria strain, which can be used as an immunogenic composition, therapeutic cargo or vaccine. For example, the immunogenic composition or vaccine can be used against a pathogenic *Salmonella* bacteria strain.

[0178] In some embodiments, the herein described DNA rescue molecule can be a plasmid vector based on the pSLT_SL1344 virulence plasmid (GenBank accession number HE654724). For example, the DNA rescue molecule can be configured to disrupt expression of one or more bacteria virulence gene(s) through plasmid incompatibility with a bacteria virulence plasmid, such as the bacteria pSLT_SL1344 virulence plasmid. For example, the DNA rescue molecule plasmid vector based on the pSLT_SL1344 virulence plasmid can be configured to include a functional auxotrophic gene (such as the *glmS* gene), thus causing retention of the DNA rescue molecule plasmid vector over the pSLT_SL1344 virulence plasmid through complementation with the disrupted chromosomal *glmS* gene.

[0179] A non-limiting example of a genetically modified *Salmonella enterica* serovar *typhimurium* is the strain (QT6282) deposited at the International Depository Authority of Canada (IDAC) under accession number 090125-01, which was deposited on January 9, 2025.

Conclusion

[0180] The methods and system described herein have led to the generation of genetically modified *E. coli* and *Salmonella* bacteria, and retention of the genetically modified phenotype without requirement of antibiotic markers. The present inventors believe that the methods and system described herein can be used on any virulent pathogenic bacteria of interest that contains a virulence plasmid. The methods and

system described herein represent a platform technology for generating genetically modified bacteria without the need for antibiotics, such as genetically modified Gram-negative bacteria.

DEFINITIONS

5 [0181] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which the present disclosure pertains. As used herein, and unless stated otherwise or required otherwise by context, each of the following terms shall have the definition set forth below.

10 [0182] The term “recombinant bacteria” broadly refers to bacteria that have been genetically modified to introduce specific genes or DNA sequences into the bacterial genome (i.e., chromosomal DNA and plasmid DNA), leading to the expression of new traits or the production of desired proteins.

15 [0183] A “mutation” is an alteration of a polynucleotide sequence, characterized either by an alteration in one or more nucleotide bases, or by an insertion of one or more nucleotides into the sequence, or by a deletion of one or more nucleotides from the sequence, or a combination of these.

20 [0184] “Genetically modified” refers to the state of the bacteria where it has been weakened from its wild-type fitness by some form of recombinant or physical manipulation. This includes altering the genotype of the bacteria to reduce its ability to cause disease. However, the bacteria’s ability to colonize the target infection tissue and induce immune responses is, preferably, not substantially compromised.

25 [0185] The term “gene” broadly refers to any segment of polynucleotide associated with a biological function. Genes can encompass both introns and exons, as seen in genomic sequences, or solely consist of coding sequences, as in cDNAs, along with the regulatory sequences essential for their expression. Additionally, the term “gene” may refer to a nucleic acid fragment that expresses mRNA or functional RNA, encodes a specific protein, and includes regulatory sequences. Genes in bacteria typically do not have introns, and as such, preferably, genes consist of coding sequences.

30

[0186] The term “conjugative transfer” refers to a process whereby DNA is transferred from donor to recipient bacteria by a specialized multiprotein complex,

termed the conjugation apparatus. A prerequisite for conjugative transfer is an intimate association between the cell surfaces of the interacting donor and recipient cells. In gram-negative bacteria, this physical contact is established by complex extracellular filaments, designated sex pili.

- 5 [0187] The term “plasmid incompatibility” refers to when two plasmids share the same replication machinery or regulatory elements, which prevents their stable coexistence within the same host cell. This phenomenon is primarily determined by the replicon region, encompassing the origin of replication (ori) and its associated regulatory components. In cases where the ori sequences of two plasmids are identical or nearly
10 identical, the plasmids typically compete for the same replication machinery within the host, resulting in incompatibility. However, minor variations in the ori sequence, such as one or more nucleotide differences, may provide a degree of flexibility depending on the nature of the changes. Specifically, such nucleotide differences may alter the recognition of the ori by replication initiation proteins, the interaction with host
15 replication factors, or other regulatory mechanisms. If these differences do not significantly affect the plasmid replication system, the plasmids may remain incompatible. The compatibility of plasmids is not solely dictated by the ori sequence but also by the specificity of the overall replication system, including initiator proteins and their interaction with the replication origin. Plasmids employing distinct replication
20 systems, such as those belonging to different incompatibility groups, are typically capable of stable coexistence, even if their ori sequences exhibit some similarity.

- [0188] Expressions such as “protection against disease,” “protective immunity,” and “functional immunity” broadly refer to a response to a disease or condition induced by the administration of one or more therapeutic compositions, or a combination thereof.
25 This response results in fewer deleterious effects than expected in a non-immunized subject exposed to disease or infection. Specifically, the severity of deleterious effects from infection is diminished in a vaccinated subject, encompassing the reduction, slowing, or potential prevention of infection.

- [0189] An “immune response” broadly refers to the development of a cellular and/or
30 antibody-mediated immune response to antigens in the composition or vaccine. The host’s display of a therapeutic or protective immunological response enhances resistance to new infection and/or reduces the clinical severity of the disease.

[0190] “Pharmaceutical- or veterinary-acceptable carrier” broadly refers to solvents, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives,

antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, etc. Stabilizers for lyophilization are preferred in certain embodiments.

- [0191] "Therapeutically effective amount" broadly refers to an amount of live genetically modified bacteria or vaccine containing the same that would induce an immune response in a subject receiving the live genetically modified bacteria or vaccine which is adequate to prevent or reduce signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a pathogen, such as bacteria. Humoral immunity or cell-mediated immunity, or both humoral and cell-mediated immunity may be induced. The immunogenic response of an animal to live genetically modified bacteria or vaccine may be evaluated, e.g., indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild-type strain. The protective immunity conferred by a live genetically modified bacteria or vaccine can be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The amount of a live genetically modified bacteria or vaccine that is therapeutically effective may vary depending on the particular live genetically modified bacteria or vaccine used or the condition of the subject and can be determined by one skilled in the art.
- [0192] "Cellular immune response" or "cell-mediated immune response" is one mediated by T-lymphocytes or other white blood cells or both, and includes the production of cytokines, chemokines, and similar molecules produced by activated T-cells, white blood cells, or both; or a T lymphocyte or other immune cell response that kills an infected cell.
- [0193] "Humoral immune response" refers to one that is mediated by antibodies. "Immune response" in a subject refers to the development of a humoral immune response, a cellular immune response, or a humoral and a cellular immune response to a live genetically modified bacteria or vaccine. Immune responses can usually be determined using standard immunoassays and neutralization assays, which are known in the art.
- [0194] "Immunologically protective amount", or "immunologically effective amount", or "effective amount to produce an immune response" of a genetically modified bacteria, immunogenic composition or vaccine is an amount effective to induce an immunogenic response in the recipient. The immunogenic response may be sufficient

for diagnostic purposes or other testing or may be adequate to prevent signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a disease agent. Either humoral immunity or cell-mediated immunity or both may be induced. The immunogenic response of an animal to an immunogenic composition may be evaluated, e.g., indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild-type strain, whereas the protective immunity conferred by a vaccine can be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The immune response may comprise, without limitation, the induction of cellular and/or humoral immunity. "Immunogenic" means evoking an immune or antigenic response. Thus, an immunogenic composition would be any composition that induces an immune response.

[0195] "Targeted knockout" is a widely used genetic engineering technique that involves the targeted disruption (e.g., removal or inactivation) of a specific gene within an organism's genome.

[0196] The term "siderophore", "iron siderophore" or "iron chelator" which are used interchangeably herein refer to compounds with high affinity for iron e.g. small compounds with high affinity for iron. Siderophores of Gram-negative bacteria are e.g. Enterobactin and dihydroxybenzoylserine synthesized by *Salmonella*, *Escherichia*, *Klebsiella*, *Shigella*, *Serratia* (but used by all enterobacteria), Pyoverdins synthesized by *Pseudomonas*, Vibriobactin synthesized by *Vibrio*, Acinetobactin and Acinetoferrin by *Acinetobacter*, Yersiniabactin and Aerobactin synthesized by *Yersinia*, Ornibactin synthesized by *Burkholderia*, Salmochelin synthesized by *Salmonella*, Aerobactin synthesized by *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia*, Alcaligin synthesized by *Bordetella*, Bisucaberin synthesized by *Vibrio*. Siderophores include hydroxamate, catecholate and mixed ligand siderophores.

[0197] The term "*Salmonella*" as used herein includes all species of *Salmonella*, including *Salmonella enterica* and *S. bongori*. The most well-known serotypes include Typhi, Paratyphi, Enteritidis, *typhimurium*, and Choleraesuis

EXAMPLES

[0198] The following examples describe some exemplary modes of making and practicing certain compositions and methods that are described herein. These

examples are for illustrative purposes only and are not meant to limit the scope of the compositions and methods described herein.

[0199] Examples 1-3 relate to a genetically modified *E. coli*, whereas Example 4 relates to an genetically modified *Salmonella*.

- 5 Example 1: Attenuation of *E. coli* by deletion of chromosomal auxotrophic and ion transporting genes

[0200] In this example, a pathogenic APEC bacteria strain was modified to generate a chromosomal disruption in an auxotrophic gene and in the Pst operon. As illustrative example, the auxotrophic gene is the *glmS* gene.

- 10 Mutation in the *glmS-pstSCA* operon

[0201] *Amplification*: A fragment of the *glmS-pstSCA operon* having the nucleic acid sequence of SEQ ID NO: 1 was amplified by PCR from an *E. coli* bacteria using the following set of primers:

- 15 CMD1347: CTGGCCGGCGCGCCCCGGTCACATGGGATGAGGAG (SEQ ID NO: 2)

CMD1349: CAGACGTTAATTAAAAGCGATGTTGTCGTAGATGG (SEQ ID NO: 3).

- [0202] *Ligation*: The resulting fragment was digested with *Ascl* and *Pacl*, and ligated into an *Ascl*-*Pacl* digested pMEG-375 plasmid (Dozois C. M., Daigle F., Curtiss R. (2002). Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. *Proc. Natl. Acad. Sci. U.S.A.* 100 247–252) to generate the allelic exchange plasmid pIJ355 (SEQ ID NO: 4). The pMEG-375 (*sacRB* *mobRP4* *oriR6K*. *Cm^R*, *Ap^R*) is a sucrose-sensitive (with the *sacRB* genetic determinant conferring sucrose sensitivity) counter selectable suicide-plasmid, which is unable to replicate in standard *E. coli* strains. A map of the allelic exchange plasmid pIJ355 is shown in FIG. 2.
- 20
- 25

- [0203] *Transformation*: The pIJ355 plasmid was then transformed into the Δasd conjugative *E. coli* donor strain $\chi 7213$ to allow replication of the pIJ355 plasmid and integration into the *glmS-pstSCA* locus by a single-crossover homologous recombination. The Δasd *E. coli* $\chi 7213$ donor strain is auxotrophic for diamino pimelic acid (DAP). The Δasd *E. coli* $\chi 7213$ donor strain being auxotrophic for DAP, this
- 30

provides an efficient postconjugational counterselection based on DAP deprivation to facilitate the transfer of plasmids.

[0204] *Selection:* Following conjugative transfer, pIJ355 integrated into the *glmS-pstSCA* locus by a single-crossover homologous recombination and selection of growth with kanamycin and no supplementation with DAP. The bacteria that were able to grow were then spread on agar plates supplemented with sucrose, kanamycin and NAG to select for a double-crossover allelic exchange.

[0205] The resulting colonies were first isolated based on their resistance to kanamycin, and because they had a BCIP-degrading phenotype that gave rise to blue colonies when BCIP was supplemented on the plates. The colonies were also screened by PCR to confirm disruption (i.e., deletion) of the *glmS-pstSCA* locus using the following set of primers:

CMD1345: AGCCCTAGGCCGCGAGACTCAGAAAGAAGG (SEQ ID NO: 5)

CMD1042: ATCGCGGTGATTGCATCAGAAAGC (SEQ ID NO: 6).

[0206] The disruption of the *glmS-pstSCA* locus was also confirmed by comparing bacteria cultured on Luria–Bertani (LB) agar plate (e.g., LB: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride), LB supplemented with N-acetyl-glucosamine (NAG), LB supplemented with BCIP, and LB supplemented with NAG and BCIP (FIG. 3A to 3D). For example, bacteria with disrupted *glmS-pstSCA* locus did not grow on plates with no supplementation of NAG.

Example 2: Preparation of *E. coli* Rescue Plasmid

[0207] In this example, a DNA rescue molecule was generated by modifying a pAPEC-1 virulence plasmid to (a) disrupt the *iuc* operon (b) disrupt the *iro* operon and (c) introduce a functional *glmS* gene therein.

[0208] The following table lists the PCR primers used in this example.

Table 0.5

Primer	Sequence	SEQ ID NO
CMD958	GAGAGAAGGCCCCGAGCGTAAACGTCTGCTG	24
CMD1042	ATCGCGGTGATTGCATCAGAAAGC	6

CMD1310	GATAAGCTTAGCATTGTTTGGCTACG	16
CMD1328	CCACCGTTTTGAAGTTCTGAG	8
CMD1329	GGGAACAGCCATTGATTGTC	9
CMD1344	GCGAAGCTTCCGTCCTGAATAGCGTTCAC	15
CMD1345	AGCCCTAGGCCGCAGACTCAGAAAGAAGG	5
CMD1346	TGCCCTAGGCTGCGGCAAGGATCACTAC	18
CMD1347	CTGGCCGCGCGCCCCGGTCACATGGGATGAGGAG	2
CMD1349	CAGACGTTAATTAAGCGATGTTGTCGTAGATGG	3
CMD1380	CGACGGTATTAATTAACCTCCGTCCTGAATAGCGTTC	20
CMD1381	GAATTCGATTTAATTAATAGCATTGTTTGGCTACG	21
Aero1	GCTCTAGATTATGATCCTGCCCTCTG	13
Aero4	ATGCATGCTGAAGCTGAGTGTACC	14

a) Disruption of the *iuc* operon

[0209] *Amplification:* Using pABN5 (McDougall S, Neilands JB) (SEQ ID NO: 7) as a template, an inverse PCR amplification of the circular plasmid was done to remove
5 the region containing the *iucABCD* genes using the following set of primers:

CMD1328 CCACCGTTTTGAAGTTCTGAG (SEQ ID NO: 8)

CMD1329 GGGAACAGCCATTGATTGTC (SEQ ID NO: 9)

[0210] *Ligation:* The linear PCR product was ligated together to generate plasmid pIJ339.

10 [0211] *Subclone into pBlueScript II:* pIJ339 was then digested with Sall and AvrII, resulting in a fragment (SEQ ID NO: 10) containing genes of interest. The fragment containing was then ligated into the pBlueScript II plasmid (Agilent Technologies) that had been digested with Sall and XbaI to generate plasmid pIJ345. The pIJ345 plasmid was then digested with BssHII resulting in a fragment containing parts of *iucA* and
15 *iucD* genes (SEQ ID NO: 11).

[0212] *Ligation:* The resulting fragment containing parts of *iucA* and *iucD* genes was ligated into the counter-selectable suicide vector pMEG-375, which had been digested with AscI, to generate plasmid pIJ346 (FIG. 4) (SEQ ID NO: 12).

[0213] *Transformation*: pIJ346 was then transformed into the DAP-requiring recipient strain (χ 7213) to serve as a donor for the transfer of the pIJ346 into APEC strain χ 7122.

[0214] *Transfer*: The pIJ346 plasmid was then transferred to APEC strain χ 7122.

- 5 Following the transfer, pIJ346, which cannot replicate in APEC strains, is then integrated into the *iucABCD* gene cluster by single-crossover homologous recombination.

[0215] *Selection*: Selected colonies were grown and spread on sucrose-containing agar plates to promote allelic exchange after double-crossover recombination. This
10 resulted in the selection of mutants having a mutated pAPEC-1 with disrupted *iucA*'-*iucD* genes that eliminate synthesis of the aerobactin siderophore through the targeted disruption of the *iuc* operon.

[0216] The double mutants were visually selected on Chrome Azurol-S (CAS)-agar plates as the yellow halo typically present around these colonies was reduced due to
15 the loss of aerobactin synthesis.

[0217] The deletion of the *iuc* genes was also confirmed by PCR (not shown) using the following set of primers:

Aero1: GCTCTAGATTATGATCCTGCCCTCTG (SEQ ID NO: 13)

Aero4: ATGCATGCTGAAGCTGAGTGTTACC (SEQ ID NO: 14).

- 20 b) Disruption of the *iro* operon and introduction of functional *glmS*

[0218] *Amplification*: The *glmU-glmS* operon was amplified by PCR from an *E. coli* bacteria to generate a *glmU-glmS* fragment (SEQ ID NO: 17) using the following set of primers

CMD1344: GCGAAGCTTCCGTCCTGA ATAGCGTTCAC (SEQ ID NO: 15)

- 25 CMD1310: GATAAGCTTAGCATTGTTTGTGGCTACG (SEQ ID NO: 16).

[0219] *Ligation*: The *glmU-glmS* fragment and the pBC-SK+ plasmid (Agilent Technologies) were then digested with HindIII and the fragment was ligated into the pBC-SK+ to generate plasmid pIJ352.

[0220] *Mutation in glmS operon (removal of glmU)*: pIJ352 was amplified by inverted PCR to remove the *glmU* gene, leaving *glmS* under the control of the *PglmU-glmS* promoter using the following primer set:

CMD1345: AGCCCTAGGCCGCGAGACTCAGAAAGAAGG (SEQ ID NO: 5)

5 CMD1346 TGCCCTAGGCTGCGGCAAGGATCACTAC (SEQ ID NO: 18)

[0221] The resulting fragment (SEQ ID NO: 19) was digested with *AvrII* and circularized by ligation to generate plasmid pIJ356.

[0222] *Amplification of mutated glmS gene*: pIJ356 was then used as a template for the amplification of the *glmS* gene and its promoter, using the following set of primers:

10 CMD1380 CGACGGTATTAATTAAGTTCCTGAATAGCGTTC (SEQ ID NO: 20)

CMD1381 GAATTCGATTTAATTAATAGCATTGTTTGGCTACG (SEQ ID NO: 21).

15 [0223] *Ligation of mutated glmS gene*: The resulting fragment (SEQ ID NO: 22) was digested with *PacI* and cloned into plasmid pIJ20 (pYA3661 - Charles M. Dozois, France Daigle, Roy Curtiss, III) that had been digested with *PvuI* to generate plasmid pIJ372.

20 [0224] *Introduction of the mutated glmS gene into pMEG-375*: pIJ372 was digested with *SphI* and the resulting *iroB*'-P_{glmS}'-*iroE* fragment was ligated into the suicide vector pMEG-375 that had been digested with *SphI*, thus generating the pIJ379 allelic exchange vector (FIG. 5) (SEQ ID NO: 23).

[0225] *Transformation*: pIJ379 was transformed into the DAP-requiring conjugative *E. coli* donor strain χ 7213 to serve as a donor for transfer of the suicide-vector.

25 [0226] *Transfer*: The pIJ379 plasmid was then transferred to the modified APEC strain χ 7122, which includes the mutated pAPEC-1 plasmid having disrupted *iucABCD* genes. Following the transfer, plasmid pIJ379 was integrated within the *iroB-iroE* cluster by a single-crossover homologous recombination.

[0227] *Selection*: Selected colonies were grown and spread on sucrose-containing agar plates to promote double-crossover recombination, leading to allelic replacement

of the *iroB-iroE* genes with a functional copy of the *glmS* gene. This resulted in the selection of mutants with disrupted *iroBCDE* genes.

[0228] The resulting colonies were also able to grow on the sucrose-containing plates without supplementation of NAG. Colonies were also visually identified by adding
5 BCIP to the growth medium. The allelic exchange resulting in deletion of *iroBCDE* genes with a *iroB'-PglmS-iroE* allele was confirmed by PCR using the following set of primers:

CMD958: GAGAGAAGGCCCGAGCGTAAACGTCTGCTG (SEQ ID NO: 24)

CMD1345: AGCCCTAGGCCGCAGACTCAGAAAGAAGG (SEQ ID NO: 5).

10 [0229] The resulting mutated pAPEC-1 plasmid ($\Delta iuc \Delta iro :: PglmS$) is devoid of functional virulence genes *iuc* and *iro*, and includes a functional *glmS* gene.

[0230] *Transfer to donor strain for future vaccine development:* The mutated pAPEC-1 plasmid vector was transferred by conjugative mating to *E. coli* laboratory strain S17 λ -*pir* to serve as the donor strain for all further transfers into strains to
15 be used as potential vaccines.

Example 3: Generation of genetically modified APEC carrying a rescue plasmid

[0231] APEC strains that have been genetically modified by the deletion of the *glmS-pstSCA* genes can be used as recipients for conjugative transfer of the plasmid vector based on the mutated pAPEC-1 plasmid of Example 2.

20 [0232] The resulting recombinant bacteria is then grown on plates without supplementation with NAG, which causes selective loss of the native pAPEC-1 virulence plasmid since both the plasmid vector and the native pAPEC-1 virulence plasmid have an identical replication system but only the plasmid vector contains the required auxotrophic gene.

25 [0233] The resulting recombinant bacteria has reduced virulence due to loss of the two siderophore-encoding systems as well as deletion of the chromosomally encoded *pst* genes.

[0234] The plasmid vector is selectively retained in the recombinant bacteria *in vivo* (since it is an environment typically poor in NAG) and on plates without
30 supplementation with NAG.

Genetically modified APEC strains – serotype O7

[0235] In this example, an APEC O7 strain was genetically modified by the deletion of *glmS-pstSCA* and the introduction of the plasmid vector based on the mutated pAPEC-1 plasmid of Example 2.

- 5 [0236] *Transfer of mutated pAPEC-1 plasmid vector to APEC O7 strain*: The plasmid vector based on the mutated pAPEC-1 plasmid ($\Delta iuc \Delta iro :: PglmS$) was transferred by conjugative transfer to an APEC O7 strain having a disruption (deletion) in the *glmS-pstSCA* genes ($\Delta glmS-pstSCA$) using the S17 λ -pir donor strain of Example 2.
- 10 [0237] *Selection*: Colonies receiving the transfer were selected by growing the bacteria on agar plates supplemented with kanamycin to counter-select against the donor strain and no supplementation with NAG to assure acquisition of the mutated pAPEC-1 plasmid vector. Thus, only recipients having received a copy of the mutated pAPEC-1 plasmid vector were able to grow on this medium. The resulting colonies
- 15 were also screened for lack of the *pstSCA* system by PCR with the following set of primers

CMD1345: AGCCCTAGGCCGCGAGACTCAGAAAGAAGG (SEQ ID NO: 5)

CMD1042 ATCGCGGTGATTGCATCAGAAAGC (SEQ ID NO: 6)

- [0238] Colonies were also identified on LB agar plate, LB + NAG, LB + BCIP, and LB + NAG + BCIP (FIG. 6A, 6B, 6C, 6D). Colonies were finally screened by PCR to confirm the presence of the mutated pAPEC-1 plasmid vector and absence of the *iuc* and *iro* genes required for synthesis of the aerobactin and salmochelin siderophores respectively (not shown).
- 20

- [0239] The final step to generate the APEC mutant vaccine strain candidates was achieved by removal of the kanamycin cassette which was flanked by FRT-containing sites and that was used to generate the *glmS-pstSCA* deletion mutation. The kanamycin resistance cassette was excised through the FLP-mediated recombination provided by the plasmid pCP20 (Cherepanov P P, Wackernagel W.). The pCP20 plasmid was then lost by heat shock following incubation at 42 °C.
- 25

- [0240] The resulting recombinant bacteria was deposited on December 21, 2023 at the International Depositary Authority of Canada (IDAC) under accession number 201223-01.
- 30

Preparation of O7 vaccine composition

[0241] An isolated colony of genetically modified O7 APEC deposited under accession number 201223-01 was inoculated in Brain heart infusion (BHI) liquid media to obtain a culture broth. On the day of vaccination, the culture broth was centrifuged at 4000 rpm for 10 min at room temperature to obtain a pellet. The pellet was suspended in phosphate buffered saline (PBS) to a final concentration of 1.3×10^{10} CFU/mL (based on optical density and CFU counting) to obtain the vaccine composition.

Efficacy evaluation of O7 *E. coli* vaccine candidate in homologous protection

[0242] 32 Specific Pathogen-Free (SPF) White Leghorn chickens of both sexes were separated into different isolators based on the group receiving the vaccine composition (Treatment 1) or the same volume of PBS (Treatment 2) on the second/third day after all birds had hatched by the oral route where the animals were flipped, which allows the vaccine strain to reach the nostrils and trachea. Chickens receiving only PBS were kept in a separate isolator to prevent cross contamination.

[0243] The chickens in the isolator 1 were vaccinated orally on day 0, with 100 μ L (1.3×10^9 CFU) of the candidate vaccine O7 strain of Example 3. The non-vaccinated group (control) received 100 μ L of PBS orally.

[0244] The chickens in the isolators were challenged 19 days after vaccination by injection of 100 μ L containing 5.00×10^7 of the virulent O7 strain directly in the left thoracic air sac.

a) Health Monitoring and Clinical Signs Observation

[0245] Chickens in all groups were monitored on specific days for any clinical symptoms (behavior, posture, eyes opening, and respiratory distress signs), and mortality until day 21. After vaccination chickens were checked on days 0 and 1 and then every 3 days until day 18. After the challenge chickens were examined on days 19, and 20 (3 times a day).

b) Necropsy

[0246] Necropsies were performed on all study chickens 2 days after the challenge to examine visible lesions in the air sacs, heart, and liver. The macroscopic fibrinous

lesions over the organs were examined according to Table 1 (Description of chicken lesions).

c) Detection of challenge strains

[0247] The level of bacteria in the blood of the chickens was determined by bacterial counts from agar plates inoculated with blood samples taken 6, 24, and 48 h after the challenge. After necropsy, the right lung, spleen, and liver were harvested and homogenized by Ultra-Turrax homogenizer. Serial dilutions of the supernatant in sterile PBS were made and each dilution was plated on MacConkey agar plates. Plates were incubated for 16 to 18 h at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

10 d) Statistical analyses

[0248] The statistical differences between lesion scores and bacterial counts recovered from blood and tissues were compared by Mann-Whitney test between vaccinated and control chicken groups using Graph Pad Prism™ 9 software (GraphPad™ Software, San Diego, CA, USA). P values ≤ 0.05 is considered significant.

Table 1

Tissues	Score	Lesion
Caudal thoracic air sac	0	Normal, clear air sac
	1	No opacity of the air sac. Blood vessels apparent
	2	Appearance of air sac opacity. Blood vessels more apparent
	3	Air sac opacity increased. Blood vessels less apparent due to fibrin presence
	4	Air sac completely opaque. Blood vessels almost not or not apparent due to fibrin accumulation
Pericardium (heart)	0	Normal, transparent, and invisible pericardium
	1	Pericardium is apparent because of its opacity. The heart is visible through it. Some fibrin accumulation on the pericardium.
	2	Pericardium is completely opaque. The heart is not visible through it. Fibrin accumulation all over the pericardium.

		Possible presence of exudate in the pericardial sac.
Liver	0	Normal, the liver is shiny and brown red
	1	Presence of fibrin covering equal or less than 25% of the surface
	2	Presence of fibrin covering 25-50% of the surface
	3	Presence of fibrin covering 50-75% of the surface.
	4	Presence of fibrin covering 75-100% of the surface

[0249] Studies of vaccine effectiveness with the genetically modified strain demonstrate that the O7 strain reduces chicken mortality when infected with the virulent strain (see, Table 2).

Survival

- 5 [0250] Morbidity started 6 h after challenge and mortality prevailed after 48 h post-challenge in the placebo group. In total, 31% (5/16) non-vaccinated birds died 48 h after challenging with the virulent O7 strain. In contrast, the vaccinated group exhibited no mortality, with all 16 birds surviving the 48-hour post-infection.

Table 2

Serotype of the vaccine strain	Mortality / Number (%)	
	Vaccinated	Non-vaccinated
O7	0/16 (0)	5/16 (31)

10

Efficacy of Vaccination

Colibacillosis

- 15 [0251] At 6 h, median level of colonization of bacteria in blood of non-vaccinated chickens was 1.32×10^3 CFU/mL while it was 5.25×10^2 CFU/mL for vaccinated (FIG. 8A, 8B, 8C). At 24 h post-infection, the titers of bacteria in blood of non-vaccinated birds were 1.92×10^3 CFU/mL compared to 45 CFU/mL in vaccinated

birds. At 48 h titers in the controls dropped to 3.43×10^2 CFU/mL, while titers in vaccinated birds were barely detectable (5 CFU/mL). Therefore, the vaccine resulted in less spread of *E. coli* to the blood stream at all time points.

5 [0252] The bacterial counts in the lungs, spleens, and livers of vaccinated chickens were significantly lower compared to those in the corresponding organs of the placebo group (FIG. 9A, 9B, 9C).

Lesion Scores

10 [0253] The Gross lesions associated with colibacillosis observed in the left air sac (site of vaccination and of infection) of infected chickens appeared to correlate with the bacterial levels detected in various tissues across all animals.

[0254] Vaccination significantly reduced the level of lesions compared to the placebo ($p=0.0110$) (FIG. 10).

Example 4: Attenuation of *Salmonella enterica* serovar *typhimurium*

15 [0255] In this example, a similarly approach as that one described in Example 1 was used to generate a genetically modified *Salmonella enterica* serovar *typhimurium*.

[0256] Colonies were screened by polymerase chain reaction (PCR) to confirm disruption (i.e., deletion) of the *glmS-pstSCA* locus in the *Salmonella enterica* serovar *typhimurium* using the following set of primers:

CDM3082: CGTTATTGGCGATGACTGCG (SEQ ID NO: 25)

20 CMD3083: GCCCGATAAATGCCGTTACC (SEQ ID NO: 26)

[0257] FIG. 16 shows that using primers CMD3082/3083 results in a 2297 bp band in the SL1344 $\Delta glmS \Delta pstSCA$ and the QT6282 strains confirming the successful deletion of *glmS* and *pstSCA* into the chromosome of SL1344. The ladder used is GeneRuler DNA Ladder Mix SM0331. If deletion is positive, then a band is visible at 25 2297 bp. If there is no deletion, then there is a band at 11538 bp.

[0258] The *glmU-glmS* promoter along with the full *glmS* gene and the *aac1* gene encoding gentamycin resistance were synthesized as a single DNA fragment via GenScript. This DNA fragment was then cloned into the *sacB*-containing allelic exchange vector pMEG-375 using the restriction sites *PacI* and *PmeI* to generate the 30 pIJ658 plasmid.

[0259] Plasmid pIJ658 was transformed into the χ 7213 DAP- strain to serve as a donor for transfer of the suicide vector to promote the allelic replacement of the *SL1344_P1_0073* gene by introduction of a functional *glmS* gene onto the pSLT_SL1344 virulence plasmid in a *S. enterica* serovar Typhimurium SL1344-
5 derivative strain that already lacked the *glmS-pstSCA* genes.

[0260] Following the transfer, plasmid pIJ658 was integrated within the *SL1344_P1_0073* gene by single-crossover homologous recombination. Selected colonies were then grown and spread on sucrose-containing plates to promote a double-crossover recombination event leading to the allelic replacement of the
10 *SL1344_P1_0073* gene with a functional copy of *glmS* gene. As such, the resulting colonies were able to grow on the sucrose-containing plates without supplementation with N-acetyl-glucosamine. The allelic exchange resulting in the deletion of *SL1344_P1_0073* gene with a *PglmS-aac1* allele was also confirmed by PCR screening.

15 [0261] The objective of this PCR was to confirm the successful insertion of *glmS* gene (Mix PCR Check *glmS* CMD3294/3295) and if the *glmS* is inserted in pSLT plasmid (Mix PCR CMD3297/3298) in the QT6282 strain.

[0262] To screen *glmS* cloned in pSLT, the following primers were used by PCR:

CDM3294: GCTGGGATATTTGGGCTCTC (SEQ ID NO: 27)

20 CMD3295: GATGTGCATGTTGTCGTTGC (SEQ ID NO: 28)

[0263] If *glmS*⁺ : Band at 592 bp. If *glmS*⁻ : No Band.

[0264] Screening of the area of insertion (*SL1344_RS24025*) was performed with the following primers by PCR:

CMD3297: ATCCGGACCGTGCCTTATAC (SEQ ID NO: 29)

25 CMD3298: ACACGCTTATCAACGACACG (SEQ ID NO: 30)

[0265] As shown in FIG. 14A and FIG. 14B, after deletion of the *glmS* gene in the SL1344 strain, the bacteria is unable to grow in media lacking N-acetylglucosamine (NAG). Indeed, the *Salmonella typhimurium* SL1344 Δ *glmS* Δ *pstSCA* does not grow on M9 media plates supplemented with BCIP (50 μ g/ml) but grows as blue colonies
30 on M9 media plates supplemented with NAG (250 μ g/mL) and BCIP (50 μ g/ml). In

contrast, the wild-type SL1344 strain grows well on M9 media plates supplemented with BCIP (50µg/ml), as shown in FIG. 15. These growth patterns demonstrate that the approach of the DNA rescue molecule and chromosomal disruption in an auxotrophic gene works well in *Salmonella*, similarly as in the case of *E. coli*.

5 Confirmation of attenuation of *Salmonella enterica typhimurium*

[0266] An isolated colony of the genetically modified *Salmonella enterica typhimurium* (QT6282) and an isolated colony of the wild type parenteral *Salmonella enterica typhimurium* QT1573 (SL1344 WT) were inoculated in LB liquid media to obtain a culture broth, which was then frozen at -80°C in 50% glycerol. On day -1 (before
10 inoculation), mice were treated with 20 mg of streptomycin in 100 µL of sterile water by oral gavage to disrupt the microbiota and facilitate the ability of SL1344 to infect the gut of C57BL/6 mice.

[0267] On the challenge day, a cryovial of the strains was thawed. Two doses (low and high) were prepared by 1:10 serial dilutions for each strain. Serial dilutions were
15 plated on MacConkey agar supplemented with 250 µg/mL of NAG, 50 µg/mL of BCIP, and 50 µg/mL of streptomycin. The plates were incubated at 37°C for 16–18 hours to confirm bacterial counts. Twenty-three healthy female C57BL/6 mice were ordered. Mice were randomly housed in separate cages and assigned unique identifiers (1 to 23).

20 [0268] Mice were divided into groups and challenged by oral gavage with either the wild-type strain (QT1573) or the mutated strain (QT6282). Two doses (low and high) were administered: Low dose groups: Used to assess mortality. High dose groups: Used for organ colonization evaluation. A control group of three mice served as the non-challenged group.

25 a) Health Monitoring and Clinical Signs Observation

[0269] Mice in all groups were monitored on specific days for any clinical symptoms (behavior, posture, eyes opening, and respiratory distress signs), and mortality until day 6 post-challenge.

b) Necropsy

30 [0270] Necropsies were performed on all study mice 6 days after the challenge. The spleen, caecum, and liver were harvested and homogenized for bacterial counts.

c) Statistical analyses

[0271] The statistical differences between bacterial counts recovered from tissues were compared by Mann-Whitney test between mice challenged with QT1573 (SL1344 WT) and mice challenged with QT6282 (SL1344 $\Delta glmS$ - $\Delta pstSCA$ - pSLT-*glmS*+) using Graph Pad Prism™ 9 software (GraphPad™ Software, San Diego, CA, USA). P values ≤ 0.05 is considered significant.

Virulence Assessment

[0272] The virulence evaluation of the QT6282 strain (*Salmonella enterica typhimurium* SL1344 $\Delta glmS$ - $\Delta pstSCA$ - pSLT::*glmS*+) demonstrated significant attenuation compared to the wild-type (WT) strain.

[0273] The genetically modified strain caused no mortality in mice, contrasting with the WT strain. Additionally, colonization levels of QT6282 in the liver and spleen were significantly lower than those of the WT strain, further substantiating its genetically modified profile, as shown in FIG. 13A to FIG. 13C.

15 Survival Analysis (Low Dose Infection)

[0274] C57BL/6 mice challenged with a low dose of the WT strain exhibited mortality starting 5 days post-challenge. In total, 3 out of 5 mice (60% mortality) succumbed within 6 days. Conversely, no mortality was observed in mice challenged with QT6282, underscoring the safety of the mutated strain, as shown in FIG. 12.

20 Organ Colonization (High Dose Infection)

[0275] The median bacteria colonization levels in organs were markedly higher for the WT strain compared to QT6282. Notably, the liver and spleen colonization levels of QT6282 were significantly lower than those of QT1573, confirming its genetically modified capacity for organ colonization, as shown in FIG. 13A, FIG. 13B, FIG. 13C.

25 [0276] Surprisingly, the QT6282 (SL1344 $\Delta glmS$ - $\Delta pstSCA$ - pSLT-*glmS*+) appears to have been genetically modified as shown with the mouse model albeit only having disruption of the *pst* gene sequences – i.e., no further disruption of one or more virulence factors.

[0277] These data suggest that virulent *Salmonella* can be genetically modified through deletion of the Phosphate Transport system genes alone and result in an attenuated *Salmonella*.

5 [0278] Other examples of implementations will become apparent to the reader in view of the teachings of the present description and as such, will not be further described here.

[0279] Note that titles or subtitles may be used throughout the present disclosure for convenience of a reader, but in no way these should limit the scope of the invention. Moreover, certain theories may be proposed and disclosed herein; however, in no
10 way they, whether they are right or wrong, should limit the scope of the invention so long as the invention is practiced according to the present disclosure without regard for any particular theory or scheme of action.

[0280] All references cited throughout the specification are hereby incorporated by reference in their entirety for all purposes.

15 [0281] Reference throughout the specification to “some embodiments”, and so forth, means that a particular element (e.g., feature, structure, and/or characteristic) described in connection with the invention is included in at least one embodiment described herein, and may or may not be present in other embodiments. In addition, it is to be understood that the described inventive features may be combined in any
20 suitable manner in the various embodiments.

[0282] It will be understood by those of skill in the art that throughout the present specification, the term “a” used before a term encompasses embodiments containing one or more to what the term refers. It will also be understood by those of skill in the art that throughout the present specification, the term “comprising”, which is
25 synonymous with “including,” “containing,” or “characterized by,” is inclusive or open-ended and does not exclude additional, un-recited elements or method steps.

[0283] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including
30 definitions will control.

[0284] As used in the present disclosure, the terms “around”, “about” or “approximately” shall generally mean within the error margin generally accepted in the

art. Hence, numerical quantities given herein generally include such error margin such that the terms “around”, “about” or “approximately” can be inferred if not expressly stated.

5 [0285] Although various embodiments of the disclosure have been described and illustrated, it will be apparent to those skilled in the art considering the present description that numerous modifications and variations can be made. The scope of the invention is defined more particularly in the appended claims.

Claims

1. A composition comprising genetically modified bacteria, wherein the genetically modified bacteria comprise:
 - a) a chromosomal disruption in an auxotrophic gene and in one or more gene(s) involved in phosphate regulation, and
 - b) a DNA rescue molecule comprising a functional copy of the auxotrophic gene,wherein the genetically modified bacteria are obtained from virulent pathogenic bacteria containing a virulence plasmid.
2. The composition of claim 1, wherein the genetically modified bacteria are Gram-negative bacteria.
3. The composition of claim 1, wherein the genetically modified bacteria belong to the Enterobacteriaceae family.
4. The composition of claim 1, wherein the genetically modified bacteria are at least one of *Escherichia coli* (*E. coli*), *Shigella*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella*, *Providencia* and *Yersinia*, preferably *Escherichia coli* (*E. coli*) or *Salmonella*.
5. The composition of any one of claims 1 to 4, wherein the chromosomal disruption includes an insertion, deletion, modification, or replacement of DNA.
6. The composition of claim 5, wherein the chromosomal disruption includes a deletion of at least a portion of the auxotrophic gene.
7. The composition of any one of claims 1 to 6, wherein the auxotrophic gene is required for bacterial cell wall peptidoglycan synthesis.
8. The composition of claim 7, wherein the auxotrophic gene is the *glmS* gene.
9. The composition of any one of claims 1 to 8, wherein the one or more gene(s) involved in phosphate regulation includes one or more gene(s) of the phosphate transport system operon (*psf* operon).

10. The composition of claim 9, wherein the one or more gene(s) of the *pst* operon is selected from *pstS*, *pstC*, *pstA*, and *pstB*.
11. The composition of claim 9 or 10, wherein the chromosomal disruption includes a deletion of at least a portion of the one or more gene(s) of the *pst* operon.
12. The composition of any one of claims 9 to 11, wherein the *glmS* gene and the one or more gene(s) of the *pst* operon are contiguous chromosomal genes.
13. The composition of any one of claims 1 to 11, wherein the DNA rescue molecule is a plasmid vector or a DNA cassette.
14. The composition of claim 13, wherein the plasmid vector contains an origin of replication incompatible with that one of the bacteria virulence plasmid.
15. The composition of claim 14, wherein the bacteria virulence plasmid includes one or more gene(s) from the siderophore system.
16. The composition of claim 15, wherein the siderophore system includes one or more gene(s) from the *iuc* operon and/or the *iro* operon.
17. The composition of any one of claims 14 to 16, wherein the plasmid vector is a copy of the bacteria virulence plasmid that has been modified with insertion of a functional copy of the auxotrophic gene in a DNA location corresponding to one or more genes of the siderophore system in the bacteria virulence plasmid.
18. The composition of claim 17, wherein the plasmid vector further includes a functional gene coding for a siderophore outer-membrane receptor, preferably the siderophore outer-membrane receptors *lutA* required for aerobactin uptake and *IroN* required for salmochelin uptake.
19. A vaccine comprising the composition of any one of claims 1 to 18 and a pharmaceutical or veterinary-acceptable excipient, diluent, or carrier, for prevention of a bacterial infectious disease.
20. The vaccine of claim 19, which is adapted for oral, ocular, transdermal, transmucosal, intravenous, subcutaneous, intramuscular, intradermal, intraperitoneal, intracapsular, intraspinal, intrasternal, intrapulmonary,

intranasal, vaginal, rectal, intraocular, intrathecal, buccal, respiratory, topical, ingestion, or local delivery administration.

21. The vaccine of claim 19 or 20, which is adapted for administration to an avian species.
- 5 22. The vaccine of claim 21, wherein the avian species is at least one of chickens, ducks, turkeys, geese, bantams, quail, pheasant, and pigeons.
23. A method for eliciting an immune response in a host, the method comprising administering to the host an effective amount of the composition of any one of claims 1 to 18.
- 10 24. The method of claim 23, wherein the composition is administered through oral, ocular, transdermal, transmucosal, intravenous, subcutaneous, intramuscular, intradermal, intraperitoneal, intracapsular, intraspinal, intrasternal, intrapulmonary, intranasal, vaginal, rectal, intraocular, intrathecal, buccal, respiratory, topical, ingestion, or local delivery administration.
- 15 25. The method of claim 23 or 24, wherein the host is an avian species.
26. The method of claim 25, wherein the avian species is at least one of chickens, ducks, turkeys, geese, bantams, quail, pheasant, and pigeons.
27. A method for preparing a live attenuated vaccine from virulent pathogenic bacteria containing a virulence plasmid, comprising
 - 20 a) causing a chromosomal disruption in the virulent pathogenic bacteria, the chromosomal disruption being in an auxotrophic gene and in one or more gene(s) involved in phosphate regulation, and
 - b) introducing in the bacteria a DNA rescue molecule comprising a functional copy of the auxotrophic gene.
- 25 28. The method of claim 27, wherein the virulent pathogenic bacteria are Gram-negative bacteria.
29. The method of claim 27, wherein the virulent pathogenic bacteria belong to the Enterobacteriaceae family.

30. The method of claim 27, wherein the virulent pathogenic bacteria are at least one of *Escherichia coli* (*E. coli*), *Shigella*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella*, *Providencia* and *Yersinia*, preferably *Escherichia coli* (*E. coli*) or *Salmonella*.
- 5 31. The method of any one of claims 27 to 30, wherein the chromosomal disruption includes an insertion, deletion, modification, or replacement of DNA.
32. The method of claim 31, wherein the chromosomal disruption includes a deletion of at least a portion of the auxotrophic gene.
- 10 33. The method of any one of claims 27 to 32, wherein the auxotrophic gene is required for bacterial cell wall peptidoglycan synthesis.
34. The method of claim 33, wherein the auxotrophic gene is the *glmS* gene.
35. The method of any one of claims 27 to 34, wherein the one or more gene(s) involved in phosphate regulation includes one or more gene(s) of the phosphate transport system operon (*pst* operon).
- 15 36. The method of claim 35, wherein the one or more gene(s) of the *pst* operon is selected from *pstS*, *pstC*, *pstA*, and *pstB*.
37. The method of claim 35 or 36, wherein the chromosomal disruption includes a deletion of at least a portion of the one or more gene(s) of the *pst* operon.
- 20 38. The method of any one of claims 35 to 37, wherein the *glmS* gene and the one or more gene(s) of the *pst* operon are contiguous chromosomal genes.
39. The method of any one of claims 27 to 38, wherein the DNA rescue molecule is a plasmid vector or a DNA cassette.
40. The method of claim 39, wherein the plasmid vector contains an origin of replication incompatible with that one of the virulence plasmid.
- 25 41. The method of claim 40, wherein the virulence plasmid includes one or more gene(s) from the siderophore system.
42. The method of claim 41, wherein the siderophore system includes one or more gene(s) from the *iuc* operon and/or the *iro* operon.

43. The method of any one of claims 40 to 42, wherein the plasmid vector is a copy of the virulence plasmid that has been modified with insertion of a functional copy of the auxotrophic gene in a DNA location corresponding to one or more genes of the siderophore system in the virulence plasmid.
- 5 44. The method of claim 43, wherein the plasmid vector further includes a functional gene coding for a siderophore outer-membrane receptor, preferably the siderophore outer-membrane receptors lutA required for aerobactin uptake and IroN required for salmochelin uptake.
- 10 45. A method for delivering a therapeutic payload in a cancer treatment, comprising administration of the composition of any one of claims 1 to 18 to a host having cancer, wherein the DNA rescue molecule further includes a recombinant gene coding for the therapeutic payload.