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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2015 (12.02.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/021147 A1

(51) International Patent Classification:
C12N 1/20 (2006.01) *A61P 31/04* (2006.01)
A61K 39/02 (2006.01)

(21) International Application Number:
PCT/US2014/049933

(22) International Filing Date:
6 August 2014 (06.08.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/862,875 6 August 2013 (06.08.2013) US

(71) Applicant: **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US).

(72) Inventors: **DHARMASENA, Madushini, Nirosha**; c/o National Institutes of Health, 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US). **KOPECKO, Dennis, J.**; 16325 Whitehaven Road, Silver Springs, MD 20906 (US).

(74) Agents: **SHERIDAN, Ross, P., C.** et al.; ATTN: NIH-OTT, 1560 Broadway, Suite 1200, Denver, CO 80202-5141 (US).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/021147 A1

(54) Title: ACID-RESISTANT, ATTENUATED MICROBIAL VECTOR FOR IMPROVED ORAL DELIVERY OF MULTIPLE TARGETED ANTIGENS

(57) Abstract: Disclosed herein is the development of an attenuated microbial vector for improved delivery of multiple targeted antigens via the insertion of cloned glutamate-dependent acid resistance genes into the attenuated microbial vector strain *Salmonella enterica* serovar Typhi strain Ty21a. The resulting Ty21a strain expresses the Gad A, B, and C proteins under an arabinose inducible promoter. In the presence of arabinose and under other acid tolerance-inducing conditions, such as growth at about pH 5.5, the Ty21a(Gad) strain tolerates exposure to pH 2.5 for 3 hrs. This acid resistance expression is sufficient for formulation of the vaccine as a wafer or tablet for human administration. Such formulation may occur without the need for buffer addition. Furthermore, such formulation enhances the ability of the immunogenic composition and/or vaccine to stimulate immune responses sublingually and throughout the intestinal tract and the ability of young children to easily swallow the composition and/or vaccine.

ACID-RESISTANT, ATTENUATED MICROBIAL VECTOR FOR IMPROVED ORAL DELIVERY OF MULTIPLE TARGETED ANTIGENS

RELATED APPLICATION DATA

[0001] Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference and may be employed in the practice of the invention. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein cited references"), as well as each document or reference cited in each of the herein cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference.

STATEMENT OF GOVERNMENT INTEREST

[0002] The instant application was made with government support; the government has certain rights in this invention.

BACKGROUND

[0003] Typhoid fever is an illness caused by the bacterium *Salmonella Typhi* and usually occurs due to consuming contaminated water or food, such as through fecal contamination of water supplies or street-vended foods. Typhoid fever is more common in the developing world, such as areas in Africa, Asia, and South America, with approximately 27 million cases and 250,000 deaths each year worldwide; 5700 cases occur annually in the US, with most acquired while traveling internationally. Symptoms include fever, headache, diarrhea, and constipation, and treatment involves appropriate antibiotic administration. However, increased resistance to antimicrobial agents has hampered disease treatment. *Salmonella Typhi* is only found in humans and is carried in the gall bladder and intestinal tract.

[0004] The mode of *S.* Typhi infection starts with oral ingestion of an infectious dose, ~ 100,000 organisms. The bacteria invade intestinal M cells in the small intestine and become engulfed by macrophages. The bacteria are then spread throughout the body by these macrophages and eventually enter the bloodstream, where an acquired immune response can be activated. *Salmonella* can reside for months or years once introduced into the gall bladder, liver, and bone marrow. Finally, the bacteria enter the bile duct and can shed sporadically into the environment through the intestines.

[0005] *Salmonella* are Gram-negative, motile, non-spore-forming rods that are members of the Enterobacteriaceae family which are characterized biochemically and by surface antigens. There are two species of Salmonella: *enterica* and *bongori*. The species enterica is further classified into 6 subspecies. The most commonly isolated is *houtenae*, followed by *diarizonae*, *salamae*, and *arizonae*. *Indica* and *S. bongori* isolates are very rare. There are over 2600 serovars with Typhi, Typhimurium, Paratyphi, and Enteriditis being some of the more common serovars. Ty2 is an isolate of *Salmonella* Typhi.

[0006] Of the more common serotypes, *Salmonella* Typhi is the causative agent of typhoid fever and is very common in under-developed countries. *Salmonella* Typhimurium is a common cause of food poisoning. The disease is not as severe as that caused by *Salmonella* Typhi. It is characterized by diarrhea, abdominal cramps, and vomiting and generally lasts up to 7 days. *Salmonella* Enteritidis is the single most common cause of food poisoning in the US. This is possibly due to mass production at chicken farms, where infection can spread from hen to hen without causing visible disease.

[0007] Vivotif or Ty21a is an attenuated mutant strain of *Salmonella* Typhi Ty2. It is the only US-licensed live oral vaccine for protection against typhoid fever. Attenuation of the strain was achieved through random mutagenesis, making the mutant $GalE^-$ and Vi-capsule negative. $GalE^-$ leads to the formation of rough LPS, which are devoid of O antigens. Galactose must be supplied exogenously to synthesize important cell wall LPS. Vivotif is highly safe, with over 200 million recipients worldwide over 30 years, and no reports of reversion to virulence and only few mild side effects observed. It has shown 70-96% efficacy in human field trails with immunity persisting completely over a full 7 years (longevity of protection has not been studied past 7 years for Ty21a).

[0008] There are many advantages to an oral live, attenuated enteric bacterial vector delivery system. The vector mimics natural infection and stimulates both mucosal and systemic immune responses. Thus, limited doses can confer long lasting immunity. The vaccine can be self-administered in a capsule form, eliminating the need for needles and

skilled heath care personnel to administer injections. There is a lower cost for vaccine manufacture, lower rate of adverse effects, higher acceptance, and it makes possible the delivery of heterologous antigens, which have been proven to be well expressed in Ty21a and stable.

[0009] The disadvantages to the Ty21a delivery system include the need to refrigerate the capsules, the requirement to take 1 hour before meals, the child-unfriendly large capsule size, and the requirement for 3 to 4 doses, each given every other day for one week. Furthermore, protection begins at 15 days.

BRIEF SUMMARY OF THE EMBODIMENTS

[0010] The licensed oral live, attenuated enteric bacterial vaccine for typhoid fever consists of the attenuated galE, Vi capsule-negative *Salmonella enterica* serovar Typhi strain Ty21a. This vaccine has a comprehensive safety record, as it has been safely administered to more than 200 million recipients worldwide over 25 years and has never reverted to virulence (Kopecko, *et al.* 2009 *Intl J Med Microbiol* 299:233-246). In addition, there has never been a documented report of post-vaccination inflammatory arthritis (*e.g.*, Reiter's syndrome), which can occur following natural infections with *Shigella, Yersinia, Campylobacter*, and non-typhoid *Salmonella* and, potentially, following administration of attenuated live vaccines containing these organisms. This vaccine also affords long-term protection (> 8 yrs) after 3-4 doses given over a 7-day period. This bacterial strain has been used extensively as a broad-based oral vaccine vector for expression of multiple foreign antigens. Ty21a has already been engineered to stably express a variety of target LPS and protein antigens to protect against shigellosis (Xu, *et al.* 2007 *Vaccine* 25:6167-6175; Xu, *et al.* 2002 *Infect and Immun* 70:4414-4423), anthrax (Osorio, *et al.* 2009 *Infect and Immun* 77:1475-1482), and plague (Kopecko, unpublished data). Ty21a induces mucosal, humoral, and cellular immunity and can be utilized as a multivalent vaccine vector that is inexpensive to produce.

[0011] *Salmonella* species encode inducible acid tolerance, but this genus does not survive well below pH 4 (Audia, *et al.* 2001 *Intl J Med Microbiol* 291:97-106). In fact, *S. Typhi* Ty21a is derived from the *rpoS* mutant Ty2 parent and is less acid resistant than Ty2. The infective dose of the Ty2 parent is about 10^6 CFU, whereas *Shigella* species require a much smaller inoculum of 10 to 500 CFU to cause dysentery, and it has been suggested that infectious doses correlate partially to the level of acid resistance of enteric pathogens (DuPont, *et al.* 1989 *J Infect Dis* 159:1126-1128; Blaser, *et al.* 1982 *Reviews Infect Dis*

4:1096-1106). *Shigella* and enterohemorrhagic *Escherichia coli* isolates have more effective acid resistance systems than Salmonella and can survive an extreme acid challenge of pH 1-2 (*i.e.*, the acidity of a human stomach when full). Thus, if a Ty21a vaccine vector can be engineered to survive very low pH for two to three hours (*i.e.*, normal transit time through a full stomach), this important modification would allow for a final delivery format for Ty21a as a rapidly dissolvable wafer, instead of the current large bullet-size enteric-coated capsule (which small children cannot swallow). Further, this wafer format would allow for immunization in the sublingual lymphoid tissue of the oral cavity, as well as throughout the gastro-intestinal tract, which should increase the overall immune response and may also reduce the doses required for long-lasting, high level protection from typhoid fever.

[0012] Ty21a exhibits a poor acid tolerance response, apparently due to the unknown mutations that occurred during random mutagenesis studies to attenuate virulence. Indeed, Ty21a does not survive well below pH 4. The inability to resist low pH reduces the ability of *Salmonella* to reach the small intestine and increases the number of cells required to initiate a successful infection. Ty21a can survive the acidic environment due to the enteric coating of the capsule in which the product is delivered. However, at least 50% of viable bacteria are lost in the encapsulation coating process, increasing vaccine costs. Delivering the vaccine in a rapidly dissolvable wafer would improve the ability of the vaccine strain to interact with host tissues (*e.g.* oral cavity lymphoid tissues) to enhance immunogenicity and acid-resistance would allow more viable attenuated *Salmonella* Typhi vaccine bacilli to reach the small intestine, possibly enhancing the immune response and possibly reducing the required CFU dose.

[0013] Further, a dissolvable wafer format would allow for active immunization in the oral cavity, as well as throughout the gastro-intestinal tract, which should enhance the overall immune response. Additionally, improving acid resistance would increase the number of viable cells that reach the small intestine and may reduce both the cfu/dose and dose number required for long-lasting, high level protection. It is also, accordingly, advantageous to minimize the losses encountered during the enteric-coating process which helps reduce the overall cost of the vaccine. Exposure to low pH prepares the invading/immunizing bacteria for the stresses of the intestine and for host-cell interactions by acting as a signal – this exposure is good as long as it doesn't kill the exposed bacteria.

[0014] Embodiments of the instant invention could be applied to many different enteric bacteria to make them acid-resistant for enhanced use as live (and possibly dead)

bacterial vaccines. In one embodiment, application of the invention improves the Ty21a oral typhoid vaccine.

[0015] Currently, Ty21a is acid-sensitive and must be delivered in an enteric-coated capsule to pass through the low pH stomach and reach the high pH of the intestine where the capsule opens and stimulates an immune response. An acid-resistant Ty21a would allow for delivery of the unencapsulated bacteria in a rapidly dissolvable wafer, which would allow for immune stimulation starting in the oral cavity, with survival through stomach acid, and more immune stimulation in the intestines. This should enhance the value of the vaccine and allow for a less expensive delivery format and increased immune response, perhaps associated with a reduced CFU/dose.

[0016] To improve Ty21a acid-resistance, the *Shigella* GDAR genes were first cloned on a low copy plasmid (pGad) under a controllable arabinose-inducible promoter. pGad enhanced the acid survival of Ty21a by 5-logs at pH 2.5 after 3 hours, when Ty21a pGad cells were pre-grown under conditions that promote an acid-tolerance response (ATR). For maximal GAD gene expression stability, the *gad* genes were inserted into the Ty21a chromosome, using a method that allowed subsequent removal of a selectable antibiotic marker and that resulted in 100% genetic stability of GAD expression. Growth of Ty21-Gad enhanced Gad protein expression and allowed for a significant 5 log-increase in viability at pH 2.5 for 3 hours compared to Ty21a alone. Further, bacterial survival assays in cultured human monocytes/macrophages indicate that the acid resistance conferred by expression of the Gad proteins in Ty21a does not alter the attenuation or safety of the vaccine. This vector enhancement should lead to an improved vaccine delivery method (*e.g.* rapidly dissolvable wafer), outside of an ECC, that will be more user-friendly and will stimulate a more robust immune response.

[0017] The survival of the original (acid-sensitive) vaccine strain Ty21a can be significantly enhanced (~5 logs) by expressing cloned *Shigella gad* genes from an inducible promoter. The growth conditions necessary for optimal acid survival of the new acid-resistant Ty21a are determined herein. This improvement enhances the value of Ty21a for use as a multivalent vaccine vector for construction of single purpose and multi-agent vaccines against numerous infectious diseases and bioterrorist agents, as well as for use in *Salmonella*-directed cancer therapy.

[0018] In one aspect, the invention provides an immunoprotective composition comprising attenuated bacteria expressing i) at least one of glutamic acid decarboxylase (Gad) proteins GadA and GadB, and ii) antiporter GadC, or functional variants thereof,

wherein the attenuated bacteria comprise an expression cassette comprising two or three glutamic acid decarboxylase (Gad) system genes GadA and/or GadB, and GadC, or functional variants thereof, isolated from *Shigella* or certain *Escherichia coli* strains (or any other organisms having an analogous GAD system) and operably linked to transcriptional promoter signals, wherein the attenuated bacteria are rendered acid-resistant as a result of the expression of the Gad decarboxylase and antiporter proteins. In a further embodiment, the immunoprotective composition further comprises a pharmaceutical diluent.

[0019] The glutamate-dependent acid- resistance system is comprised of two decarboxylases and an antiporter. Either or both decarboxylases convert glutamate to GABA, which sops up one H+ ion in the process and is transported out of the cell by GadC, which picks up a new glutamate and brings it into the cell. This process repeats itself until the cell gets close to neutral pH, when the GadC pore closes and doesn't reopen until the pH becomes acidic.

[0020] Addition of the Gad cassette, under a controllable promoter, to many different organisms may enhance their value as probiotics, food-based organisms, or various environmentally useful organisms, where acid-resistant enhancement would be valuable.

[0021] In one embodiment, the lysine decarboxylase gene system enhances the Gad-induced acid resistance.

[0022] In one embodiment of a composition or vaccine or method according to the invention, the attenuated bacteria are selected from the group consisting of *Campylobacter jejuni, Campylobacter coli, Listeria monocytogenes, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis. Escherichia coli, Shigella flexneri, Shigella sonnei, Shigella dysenteriae, Shigella boydii, Helicobacter pylori, Helicobacter felis, Gastrospirillum hominus, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Bacteroides fragilis, Clostridium difficile, Salmonella typhimurium, Salmonella typhi, Salmonella gallinarum, Salmonella pullorum, Salmonella choleraesuis, Salmonella enteritidis, Salmonella spp., Streptococcus gordonii, Lactobacillis sp., Klebsiella pneumoniae, Enterobacter cloacae,* and *Enterococcus faecalis.*

[0023] In another embodiment of a composition or vaccine or method according to the invention, the attenuated bacteria are selected from the group consisting of *Salmonella typhi* Ty21a, *Vibrio cholerae* CVD103HgR, and *Vibrio cholerae* Peru15.

[0024] In yet another embodiment of a composition or vaccine or method according to the invention, the attenuated bacteria are probiotic bacteria selected from the group consisting of actinobacteria, proteobacteria, firmicutes, and bacteroidetes (De Biase, *et al.*

2012 *Molec Microbiol* 86(4):770-786). In still another embodiment, the probiotic bacteria are selected from the group consisting of *Bifidobacterium*, *Lactobacillus spp.*, *Escherichia coli*, and *Lactococcus lactis*.

[0025] Such probiotic bacteria are currently packaged in capsules, but may be made acid-resistant by inserting the glutamic acid decarboxylase system (Gad) genes described herein and could then be administered in a "simpler" format. These organisms may contain native acid-resistance systems under complex regulatory control, which could be enhanced in value by adding cloned Gad genes under a controllable promoter.

[0026] Thus, the cloned GAD system (under a controllable promoter and on a movable cassette) can, in certain embodiments, be added to probiotic bacteria or live vaccine strains, i.e., strains that have GAD controlled by a more complex regulatory network, to stimulate resistance to low pH, *i.e.*, acid resistance, which is advantageous for final delivery formats, e.g. allowing the organism to be taken out of enteric capsules and presented in a "simpler" format, such as rapidly dissolving wafers or membranes.

[0027] In another embodiment of a composition or vaccine or method according to the invention, the expression cassette comprises SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5 or a functional variant thereof, each operably linked to a promoter or at least two or all three linked together and operably linked to a promoter. In a further embodiment, the promoter is an arabinose-inducible promoter. In still a further embodiment, the promoter is a constitutive promoter. In yet other embodiments, a different promoter is contemplated as useful for expression of the GAD genes.

[0028] In another embodiment of a composition or vaccine or method according to the invention, the Gad proteins are expressed from a recombinant plasmid. In a further embodiment, the recombinant plasmid contains at least one selectable marker. In still another embodiment, a kanamycin resistance gene flanked by FRT sites could be used for selection (for example, in Ty21a); then the selectable marker can be removed by recombination, leaving the Gad genes on a plasmid without antibiotic resistance.

[0029] In another embodiment of a composition or vaccine or method according to the invention, the Gad proteins are expressed from a chromosomal integration site.

[0030] In one aspect, the invention provides a plasmid comprising a polynucleotide encoding the genes i) *GadA* and/or *GadB*, and ii) *GadC*, or functional variants thereof, from a *Shigella* species operably linked to transcriptional promoter signals. In a further embodiment, the plasmid encodes glutamic acid decarboxylase (Gad) proteins i) GadA and/or GadB, and ii) GadC, or functional variants thereof.

[0031] In one aspect, the invention provides acid-resistant attenuated bacteria, wherein the bacteria express: i) at least one of glutamic acid decarboxylase (Gad) proteins GadA and GadB, and ii) antiporter GadC, or functional variants thereof, wherein the attenuated bacteria comprise an expression cassette comprising two or three glutamic acid decarboxylase system (Gad) genes *GadA* and/or *GadB*, and *GadC*, or functional variants thereof, isolated from a *Shigella* species or *Escherichia coli* (or other organism having an analogous GAD system) and operably linked to transcriptional promoter signals, wherein the expression of the three Gad proteins renders the attenuated bacteria acid-resistant. In a further embodiment, the bacteria are induced by growth in Tryptic Soy Broth at about pH 5.5 in the presence of about 0.75% arabinose.

[0032] In one aspect, the invention provides a method for preparing acid-resistant attenuated bacteria, comprising growing bacteria comprising an expression cassette comprising two or three glutamic acid decarboxylase (Gad) genes *GadA* and/or *GadB*, and *GadC*, or functional variants thereof, isolated from a *Shigella* species or *Escherichia coli* and operably linked to transcriptional promoter signals in a culture medium having subneutral pH under aerobic culture conditions. In a further embodiment, the subneutral pH lies between about pH 4.0 and about pH 6.8. In still a further embodiment, the subneutral pH lies at about 5.5. In additional embodiments, the culture medium comprises at least one of glucose, arabinose, and glutamate.

[0033] In one aspect, the invention provides a vaccine comprising an immunoprotective composition as described herein and a pharmaceutically acceptable carrier. In another embodiment, the vaccine is formulated as a compressed wafer, tablet, dissolvable membrane, or chewable gum. In still another embodiment, the compressed wafer, tablet, dissolvable membrane, or chewable gum comprises bicarbonate. In still another embodiment, the bicarbonate is in an amount lower than the amount that would be required for full buffering of the stomach. In yet another embodiment, the compressed wafer, tablet, dissolvable membrane, or chewable gum is formulated with arabinose and glutamate.

[0034] Additional embodiments of the invention are described and contemplated herein, as set forth, without limitation, in the remainder of the instant application.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Figure 1A is a schematic representation of the following: the three gad genes, gadA, gadB, and gadC were cloned in tandem with their own ribosome binding sites under

the control of an arabinose inducible promoter in a low copy plasmid pNW129 (Bonocora, *et al.* 2008 *Molec microbiol* 69:331-343) to express these genes with arabinose induction and, thus, free from the typical complex regulatory network. Figure 1B shows the expression of Gad proteins as determined by western blot with anti-Gad A/B antibody. Gad A and Gad B were 98% similar at the protein level. The cloned GadA/B were not expressed in the absence of arabinose induction, but Gad A/B were expressed visibly as early as 2 hours after arabinose induction, and their expression increased over time. Gad A/B were not expressed in the negative Ty21a control with the empty plasmid vector, but were expressed in overnight cultures of positive control *S. flexneri* strain CFS100.

[0036] Figures 2A and 2B: in determining that extreme acid survival of bacteria depends on growth phase, when tested bacteria were pre-grown to log phase in commercial TSB (Figure 2A) or TSB-MES pH 5.5 (Figure 2B), Ty21a with pGad (Ty21a pGad), Ty21a with empty vector (Ty21a pNW129), or wildtype S. Typhi Ty2 did not show any survival at pH 2.5 after 3 hours. In contrast, *S. flexneri* (*Sf*) showed ~0.01% survival in media at pH 2.5 after 3 hours. Figure 2C shows, however, that when bacteria were grown to stationary phase (*i.e.*, for 18 hours) in regular TSB prior to pH 2.5 acid challenge, Ty21a pGad showed ~5 log increase in acid survival compared to the negative control Ty21a pNW129 after 3 hours (*i.e.*, comparable to survival of *Shigella flexneri*, *Sf*). Surprisingly, under these conditions Ty2 did not show any survival. Importantly, the ~5 increase in acid survival of Ty21a pGad was dependent upon media supplementation with arabinose. Figure 2D: when tested bacteria were pre-grown to stationary phase in TSB-MES pH 5.5 prior to the pH 2.5 acid challenge, Ty21a pGad, Ty2 and *Sf* showed good acid survival, whereas Ty2la pNW129 demonstrated poor acid survival (*i.e.*, ~5 logs less than that of the other strains).

[0037] Figure 3 graphically depicts how extreme acid survival of *S.* Typhi strains depends upon the pH of the pre-growth media. Tested bacteria were pre-grown in either TSB-MES pH 4.5, TSB-MES pH 5.5, TSB-MOPS pH 6.5, commercial TSB (unbuffered pH 7.2), or TSB-MOPS pH 7.5 for 18 hours prior to the acid challenge, and survival at pH 2.5 for 3 hours was determined. Ty21a with pGad showed good survival when bacteria were pre-grown in mildly acidic pH media and in commercial unbuffered TSB. However, following pre-growth at pH 7.5, acid survival was decreased by ~ 3 logs. It is plausible that the ATR systems of Ty21a are not induced at pH 7.5, and that the induction of ATR in addition to the Gad system may be important for optimal acid-survivability. The negative control Ty21a pNW129 did not show any survival after any of the pre-growth conditions. Although Ty2 showed good low pH-survival when pre-grown at slightly acidic pH, it showed very poor

survival when pre-grown at pH 7.5 or in commercial TSB at pH 7.2, indicating that the ATR of Ty2 is activated only at mildly acidic pH. Sf showed good survival under all pre-growth conditions, including pre-growth in TSB-MOS pH 7.5. Thus, unlike *S.* Typhi, acid survival of *Shigella* does not require mildly acidic pre-growth conditions.

[0038] Figure 4A provides results in bar graph form for the following experiment: bacteria were grown in commercial TSB supplemented with 0.25% glucose or TSB without added glucose for 18 hours, and the pH of the cultures was determined. The pH of Ty21a pGad and Ty21a pNW129 cultures dropped below 6.5 after 18 hours, whereas the pH of the Ty2 culture remained close to pH 7.2. The pH of the Sf culture dropped even further, to below pH 6. However, in the absence of added glucose to TSB, the pH drop was not observed with Ty21a strains, indicating that glucose is responsible for the pH drop. The pH drop of Sf cultures was not dependent on glucose in the media. Figure 4B provides a graph showing acid survival of bacteria compared in the presence or absence of glucose in commercial TSB (pH 7.2). Acid survival ability of Ty21a pGad was greatly diminished (~ 3 logs after 3 hours) when the bacteria where grown in TSB without glucose. This indicates that Ty21a pGad pre-growth in media supplemented with glucose is important for acid survival by acidifying the media and/or providing an energy source for activation of ATR. Figure 4C provides a graph showing acid survival of tested bacteria compared in the presence and absence of glucose in TSB-MES pH 5.5. Acid survival ability of Ty21a pGad and Ty2 were significantly reduced (4 to 5 logs after 3 hours) when glucose was absent in the media. Even Ty21a acid survival ability was reduced (~ 3 logs after 1 hour), indicating that pre-growth in glucose is important to activation of ATR in *S.* Typhi strains, in addition to acidifying media of Ty21a cultures. However, *Sf* acid-survival was not affected by presence or absence of glucose in the media. Thus, glucose in the pre-growth media is important for extreme acid survival of *S.* Typhi strains under these conditions.

[0039] Figure 5 schematically depicts the integration of Gad genes into the Ty21a chromosome using a suicide vector. The R6K-based suicide vectors, pMD-SV(upper schema) and pMD-SD (lower schema), which require a trans-supply of pir-encoded pi protein for replication, were constructed. Although these plasmids can replicate in strains that express pi protein such as S17 λpir, efficient plasmid suicide results upon transfer to Ty21a, which does not encode pir. Strain S17λpir carrying a KanR pMD-SV, which contains homology to the Vi operon vexA gene, was conjugated with Ty21a to transfer the pMD-SV plasmid. Kanamycin-resistant Ty21a with pMD-SV integrated into the Vi operon were first selected. Next, the region containing the KanR gene between the FRT (flippase recognition

target) sites was eliminated by expression of the flippase enzyme from replication temperature-sensitive plasmid pCP20, resulting in the vexA merodiploid strain MD290. The gad genes under control of an arabinose promoter were cloned into pMD-SD (pMD-SD-gad), integrated into vexA merodiploid MD290, and the region between FRT sites was eliminated to construct the KanS Ty21a-Gad (MD297).

[0040] Figure 6A provides an immunoblot depicting expression results from the following: selected bacteria were grown in TSB-MES pH 5.5 with or without glucose for 18 or 24 hours prior to acid challenge. Although Ty21a-Gad did not express detectable Gad proteins at 18 hours in the presence of glucose, Gad-expression was observed at 24 hours. Interestingly, Gad proteins were expressed even in the absence of glucose after 18 hours. In contrast to single copy Ty21a-Gad, the plasmid construct (Ty21a pGad) existed at ~ 5 copies per cell and expressed a higher level of Gad proteins under all conditions. Figure 6B graphically shows that, consistent with level of Gad protein expression, Ty21a-Gad acid survival was comparable to that of Ty21a after 18 hours of growth , but acid survival improved significantly (~3 logs) when Ty21a-Gad were grown for 24 hours prior to acid challenge. Thus, chromosomal integrant (Ty21a-Gad) can survive extreme low pH when pre-grown for 24 hours under appropriate inducing conditions.

[0041] Figure 7 shows a bar graph depicting survival in cultured human monocyte/macrophage cells as a key virulence trait of wildtype *S.* Typhi, but that is mutated in Ty21a. Macrophage survival of strains expressing Gad proteins (Ty21a pGad and Ty21a-Gad), Ty21a, and Ty2 were performed as previously described (Dragunsky, *et al.* 1989 *J biol standardization* 17: 353-360). The % survival was calculated as the ratio of bacteria that survived and replicated inside cultured human U937 macrophage cells 24 hours after infection, as a fraction of the bacteria that invaded the macrophage cells (4 hours). Ty21a pGad and Ty21a-Gad showed very low survival, comparable to Ty21a, whereas Ty2 showed robust replication within macrophages, suggesting that expression of Gad proteins in Ty21a does not alter its attenuation. Thus, Gad-induced acid resistance in Ty21a does not alter strain attenuation, as determined by survival in macrophages.

DETAILED DESCRIPTION

[0042] The ability of enteric bacteria, after ingestion, to withstand acid stress in the stomach is crucial for successful colonization in the gastrointestinal tract. Each microorganism exhibits a different degree of resistance to acid stress, which involves acid

tolerance response (ATR) systems and/or acid resistance (AR) systems. The AR systems typically protect stationary-phase cells from an extreme acid stress without pre-adaptation. They "mop up" the H^+ that leak into the cytoplasm. AR systems generally consist of a decarboxylase enzyme that is activated by low pH in the presence of a specific amino acid and an antiporter. Five bacterial AR pathways, AR1-AR5, have been described previously.

[0043] AR1 is the stationary phase acid-induced, glucose-repressed oxidative pathway. AR1 is dependent on the RNA polymerase sigma factor rpoS. This can be found in *E. coli* and *S. flexneri*. AR2-AR4 are all amino acid decarboxylase-based pathways. The decarboxylase enzyme is induced by low pH and the presence of the specific amino acid and an antiporter. AR2 and AR3 enable bacteria to survive in extreme acidic environments (pH 2.5), while AR4 enable bacteria to survive moderately acidic environments (pH 4.5). AR2, the acid resistance pathway of interest here, encompasses the highest level of acid resistance. It is the stationary phase, glutamate-dependent acid resistance pathway. It is made up of two glutamate decarboxylases, GadA and GadB, and an inner-membrane antiporter, GadC. This system is typically encoded by certain *E. coli* strains (e.g. enterohemmorhagic *Escherichia coli 0157*) and Shigella *strains (e.g. Shigella flexneri),* as well as *Listeria monocytogenes* and *Lactococcus lactis*, but not *Salmonella* serovars. With multiple acid resistance pathways such as AR1 and AR2, cells can survive below pH 2.5.

[0044] AR3 is the stationary phase arginine-dependent acid resistance pathway, found in *E. coli*, *S.* Typhi, and *S.* Typhimurium. In *S.* Typhimurium, AR3 is induced only in the absence of oxygen. AR4 is the lysine decarboxylase acid resistance pathway, also found in *E. coli*, *S.* Typhi, *S.* Typhimurium, and *Vibrio cholerae*. AR5, found in *E. coli*, consists of an inducible decarboxylase that has not, to date, been well studied. (De Biase, *et al.* 2012 *Molec microbiol* 86:770-786; Zhao, *et al.* 2010 *Biochem and cell biol* 88:301-314; Brenneman, *et al.* 2013 *J Bacteriol* 195:3062-3072; Merrell, *et al.* 2002 *Curr Opin microbiol* 5:51-55). There are also acid tolerance responses in *Salmonella*, including log phase responses, in which exponential cells use acid shock proteins to become adapted to a moderately low pH (pH 4.5-5.8) and, stationary phase responses, in which cells can survive to pH 3 after being exposed to subneutral mild pH (below pH 5). Exposing cells to acid stress also provides cross-protection against many other environmental challenges such as oxidative stress, heat shock, high osmolarity, and DNA damage, but it is not reciprocal, and these other stresses do not cross-protect against acid stress.

[0045] ATR systems, in contrast, comprise acid-stress responses that result in the induction of a set of proteins called acid shock proteins (ASPs), that are typically induced at

mildly acidic pH, and that prepare the cell for potential extreme acidic pH exposure. ATR has been mostly described in *S.* Typhimuirum. The ASPs presumably act to repair and prevent formation of damaged macromolecules caused by acid stress. For example, the ASPs include DNA repair enzymes *polA* and *ada,* which repair acid-damaged DNA. The ATR also shifts the fatty acid profile of the cell, contributing to increased hydrophobicity and thus decreasing proton influx across the membrane. Key proteins that regulate the subset of ASPs include: alternative sigma factor RpoS; iron regulator, Fur; and two component regulatory systems such as PhoPQ and OmpR/EnvZ (Foster, *et al.* 1995 *Critical rev microbiol* 21: 215-237; Audia, *et al.* 2001 *IJMM* 291: 97-106; Foster 1999 *Current opinion microbiol* 2:170-174; Foster, *et al.* 1994 *J bacteriol* 176: 2596-2602). Of note, the *S.* Typhi vaccine strain Ty21a and other live, attenuated *S.* Typhi vaccine candidates derived from the Ty2 parent are *rpoS* mutants (Robbe-Saule, *et al.* 1999 *FEMS microbiol lett* 170: 141-143). However, Ty21a is more acid-sensitive than Ty2. Apparently, some mutation(s) that were introduced by random mutagenesis during virulence attenuation (Germanier, *et al.* 1975 *J infec dis* 131: 553-558) further reduce Ty21a ability to survive at low pH. In fact, Ty21a contains 679 single nucleotide polymorphisms (SNP) introduced during random mutagenesis (Xu, *et al.* 2013 *Genome announcements* 1; Hone, et al. 1994 *Vaccine* 12: 895-898).

[0046] Unlike *Shigella*, *Salmonella* do not have a glutamate-dependent acid resistance (GDAR) system. Although *Salmonella* encode inducible ATR, AR3, AR4, and AR5 systems, this genus is only moderately acid-resistant (Viala, *et al.* 2011 *PloS one* **6**: e22397). Indeed, it does not survive well below pH 4. The infectious dose of *Salmonella* Typhi is estimated at 10^3 to 10^6 CFU, depending on the growth environment (Blaser, *et al.* 1982 *Reviews infec dis* 4:1096-1106; Hornick, *et al.* 1966 *Transactions Assoc Amer Physicians* 79:361-367), whereas *Shigella* species require a much smaller inoculum of 10 to 500 CFU to cause dysentery (DuPont, *et al.* 1989 *J infec diseases* 159:1126-1128); and it has been suggested that infectious dose correlates strongly with the level of acid resistance of enteric pathogens (Audia, *et al.* 2001 *IJMM* 291:97-106).

[0047] *Shigella* species possess at least two acid resistance pathways. Acid resistance pathway I (ARI) is the stationary phase, acid-induced, glucose-repressed oxidative pathway that protects these bacteria above ~pH 3.5, while acid resistance pathway 2 (AR2) is the stationary phase, glutamate-dependent acid resistance (GDAR) pathway that protects *Shigella* down to pH 1. The GDAR acid resistance pathway is normally absent in *Salmonella.* The 3 *Shigella* genes for GDAR are well-characterized. GDAR requires 2 glutamate decarboxylases (encoded by paralogous genes *gadA* and *gadB*) and the antiporter GadC,

which exchanges intracellular decarboxylated glutamate with extracellular glutamate to maintain pH homeostasis. These genes are typically under the control of a complex network of genetic regulation (Lin, *et al.* 1995 *J Bacteriol* 177:4097-4104); Bhagwat 2004 *FEMS Microbiol Lett* 234:139-147).

[0048] The 3 *Shigella* GDAR genes were cloned on a low copy plasmid (pNW129) in Ty21a under the control of an arabinose-inducible promoter to allow for full Gad expression in the presence of arabinose (*i.e.*, the newly developed strain is termed Ty21a-pGad). The strains, Ty21a-pGad and Ty21apNW129 (empty vector), were grown in Tryptic Soy Broth (TSB) at pH 5.5 for ~16 hours until the cells reached stationary phase. Ty21a-pGad was shown by Western blot with Gad-specific antibodies to be synthesizing significant quantities of the Gad proteins, as expected. Next, these stationary phase cells were diluted 1:20 in TSB at pH 2.5 for 2 hrs, (*i.e.*, acid shock treatment meant to mimic passage through the stomach), and the survival was monitored by plating dilutions on TS agar. After 2 hours at pH 2.5, Ty21a expressing the Gad proteins (determined by Western blot) showed 50% survival, compared to Ty21a with the empty vector showing a 2-log drop in viability, demonstrating that expression of the Gad proteins in Ty21a does improve its acid resistance under selected growth conditions.

[0049] Glutamic acid decarboxylase (Gad) is only found in some *E. coli* species, *i.e.*, those that are able to resist low pH, and in *Shigella* species. Glutamate-dependent acid resistance works by mopping up protons leaking into the bacterial cytosol through the decarboxylation of glutamate to gamma-aminobutyric acid (GABA). GABA is transported extracellularly in exchange for external glutamate by GadC to maintain pH homeostasis intracellularly. Thus, the pH increases with the consumption of H+. Under acidic conditions, external glutamate is taken into the cell and converted to GABA by the action of GadA and GadB, producing CO2. GABA+ is exported out of the cell in exchange for more glutamate to continue this process. However, under neutral conditions, a GadC-plug blocks the exchange of glutamate and GABA, maintaining the pH homeostasis of the cell.

[0050] The pH-dependent transport activity of GadC ensures that substrate exchange can be efficiently activated only in an acidic environment, preventing an unnecessary waste of energy.

[0051] In one embodiment, this invention is directed to a living, attenuated, oral vaccine capable of inducing an immunoprotective response against different microbial, viral, or protozoan antigens or toxic molecules (*e.g.* ricin or botulinum toxin). The invention is based on an attenuated strain of bacteria which has been genetically engineered to carry three

GDAR acid resistance (gad) genes. These recombinant bacteria are useful in an immunoprotective composition to induce an immunoprotective response in a susceptible host organism. Enteric infections caused by bacterial and certain microorganisms or intoxication due to exposure to toxins (*e.g.* ricin) are considered amenable to treatment with a vaccine according to this invention. For example, genes encoding the key protective surface antigens derived from *Shigella* strains such as *S. flexneri, S. dysenteriae*, and *S. sonnei* (see *e.g.*, Baron, *et al.* 1987 *Infect and Immun* 55:2797) can be transferred into recipient vaccine vector bacteria. The resulting recombinant live, attenuated vaccine can then express two or more heterologous surface antigens suitable for generating an immunoprotective response in a host organism. Alternatively, the oral vaccine may contain a mixture of multiple strains of attenuated bacteria, each strain expressing a different heterologous antigen. This resulting vaccine would also be suitable for generating an immunoprotective response against multiple antigens in a host organism.

[0052] Genes encoding other antigens, such as genes encoding non-toxic variants of toxins derived from enterotoxogenic (*i.e.*, heat-labile LT or heat-stable ST toxins)or enterohemmorhagic (*i.e.*, Shiga toxins) strains of *Escherichia coli*, or protective antigens from *Vibrio cholera*, *Yersinia pestis* (plague F1 and V antigens) and *Bacillus anthracis* protective antigen PA or toxoid/toxin lethal factor or edema factor can also be transferred into bacterial host vaccine vectors. In one embodiment, the non-toxic variants of the enterotoxins should be expressed in such a way that the proteins are present on the surface of the recombinant bacteria or secreted by the recombinant bacteria. The resulting recombinant bacteria would be useful in immunogenic compositions for generating an immunoprotective response to these antigens. Enteric disease caused by bacterial secretion of an exotoxin exemplified by staphylococcal, clostridial or similar food poisoning/intestinal agents are also considered amenable to treatment with an immunoprotective composition according to this invention using an approach similar to the approach used for enterotoxins.

[0053] Nucleic acids encoding the *gad* genes may be cloned into vectors for transformation into bacterial cells for replication, expression, and cell transformation. Such vectors are typically prokaryotic vectors, *e.g.*, plasmids that act as shuttle vectors, or for production of protein. The elements that are typically included in vectors include a replicon that functions in the recombinant bacteria, a gene encoding a selectable marker to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of recombinant sequences. Selectable markers may include a gene encoding antibiotic resistance, or may include a gene encoding a

protein whose naturally occurring gene has been mutated resulting in an attenuated strain of bacteria. Examples of suitable targets for mutation include genes that would result in essential auxotrophic pathways, loci encoding regulons that exert pleiotropic effects such as the cya/crp system, the ompR/envZ system or the phoP system (see *e.g.* U.S. Pat. Nos. 5,672,345, 5,980,907, 6,190,669). A preferred selectable marker is the aspartate β-semialdehyde dehydrogenase (*asd*) gene operably linked to a promoter. A recombinant plasmid capable of expressing asd could complement the asd phenotype of attenuated bacterial strains suitable for use in vaccines and containing asd deletion mutations. Bacteria lacking asd would not be able to synthesize diaminopimelic acid, an essential element of the peptidoglycan of the bacterial cell wall, and would die. Examples of other selectable markers useful in bacteria include SacB, aroA, and heavy metal ion resistance genes.

[0054] Alternatively, the vectors may be transformed into bacterial cells carrying a mutation in the msbB gene, which attenuates by knocking out the main component of endotoxin. Mutations in this gene fail to myristylate lipid A and have reduced endotoxicity. Bacteria containing this mutation may contain additional mutations resulting in attenuated bacteria and vectors containing the genes that produce acid resistance and may contain selectable markers. Gad production in bacteria containing a mutation in the msbB gene may allow the strain to be used to express other foreign antigens by techniques well known to those of skill in the art and used in an immunoprotective composition directly.

[0055] To obtain expression of the *gad* genes, the nucleic acids encoding the appropriate gene(s) are typically subcloned using techniques well known to those of skill in the art, into an expression vector that contains a promoter to direct transcription. Suitable bacterial promoters are well known in the art and described, *e.g.* in Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1989); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., eds., 2001).

[0056] The promoter used to direct expression of the genes depends on the particular application. Either a constitutive or an inducible promoter may be used. In one embodiment, an arabinose-inducible promoter is used.

[0057] The promoter can also include elements that are responsive to transactivation, e.g., hypoxia response elements, Gal4 response elements, lac repressor response element, and small molecule control systems such as tet-regulated systems and the like (see, *e.g.*, Gossen & Bujard 1992 *PNAS USA* 89:5547; Oligino, *et al.* 1998 *Gene Ther* 5:491-496; Wang, *et al.*

1997 *Gene Ther* 4:432-441; Neering, *et al.* 1996 *Blood* 88:1147-1155; and Rendahl, *et al.* 1998 *Nat Biotechnol* 16:757-761).

[0058] In addition to the promoter, the expression vector may contain a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in recombinant bacteria. A typical expression cassette thus may contain at least one promoter operably linked, *e.g.*, to the nucleic acid sequences encoding the *gad* genes, and signals required, *e.g.*, for transcriptional termination, ribosome binding sites, or translation termination. Additional elements of the cassette may include, *e.g.*, regulatory proteins.

[0059] Standard bacterial vectors include plasmids such as pBR322-based plasmids, pBR325, pUC18, pSKF, pET23D, and pBR322 based-cosmid vectors such as pHC79 and pCVD551. Vectors based on the bacterial plasmid pSC101 such as pGB-2 may also be used. Further, the *gad* gene cassette can be inserted into the chromosome of the desired strain by methods already described (Dharmasena, *et al.* 2013).

[0060] Standard transformation methods are used to produce bacterial cell lines that express the Gad proteins of the invention. Transformation of prokaryotic cells is performed according to standard techniques (see, *e.g.*, Morrison 1977 *J Bact* 132:349-351; Sambrook, *et al.*, *supra*; Ausubel, *et al.*, *supra*). The methods include microinjection, ballistics, use of calcium chloride transformation, infection, conjugation, and electroporation of plasmid vectors, both episomal and integrative, and any of the other well-known methods for introducing cloned genomic DNA, synthetic DNA or other foreign genetic material into a recombinant bacteria (see, e.g., Sambrook, *et al.*, *supra*, see also U.S. Pat. No. 5,049,386, U.S. Pat. No. 4,946,787; U.S. Pat. No. 4,897,355; WO 91/17424, and WO 91/16024). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing a Gad cassette into the recombinant bacteria capable of expressing the Gad proteins.

[0061] The microorganisms that are used to express the *gad* genes for use in immunoprotective compositions include without limitation, *Campylobacter* sp., *Yersinia* sp., *Helicobacter* sp., *Gastrospirillum* sp., *Bacteroides* sp., *Klebsiella* sp., *Lactobacillus* sp., *Streptococcus* sp., *Enterobacter* sp., *Salmonella* sp., *Shigella* sp., *Aeromonas* sp., *Vibrio* sp., *Clostridium* sp., *Enterococcus* sp. and *Escherichia* sp.(see e.g. U.S. Pat. Nos. 5,858,352, and 6,051,416, and Levine, *et al.* 1997 "New Generation Vaccines Second Edition" ed. Levine, *et al.*, Marcel Dekker, Inc. pp 351-361, Levine, *et al.* 1997 "New Generation Vaccines Second Edition" ed. Levine, *et al.*, Marcel Dekker, Inc. pp 437-446, Butterton, *et al.* 1997 "New

Generation Vaccines Second Edition" ed. Levine, *et al.*, Marcel Dekker, Inc. pp 379-385 , and Fennelly, *et al.* 1997 "New Generation Vaccines Second Edition" ed. Levine, *et al.*, Marcel Dekker, Inc. pp 363-377).

[0062] Preferred enteric bacteria that the various aspects of the present invention relate to are *Campylobacter jejuni, Campylobacter coli, Listeria monocytogenes, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Escherichia coli, Shigella flexneri, Shigella sonnei, Shigella dysenteriae, Shigella boydii, Helicobacter pylori, Helicobacter felis, Gastrospirillum hominus, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Bacteroides fragilis, Clostridium difficile, Salmonella typhimurium, Salmonella typhi, Salmonella gallinarum, Salmonella pullorum, Salmonella choleraesuis, Salmonella enteritidis, Klebsiella pneumoniae, Enterobacter cloacae*, and *Enterococcus faecalis*. In additional embodiment, the Gad genes can be inserted in beneficial probiotic bacterial strains including, but not limited to, *Lactobacillus*, *Bifidobacterium*, *Streptococcus spp.*, and the like to allow for acid resistance expression leading to administration outside of an enteric-voated capsule.

[0063] More preferred strains of *Escherichia* coli include DH5α and HB101. More preferred strains of *Salmonella typhi* include CVD 908, CVD 908-htrA, X4073 and TY800.

[0064] Most preferred strains of bacteria to use as live attenuated vaccines include *S.* Typhi, strain Ty21a, which carries a mutation in its galE gene, and *V. cholerae* carrying mutations in its ctxA gene which prevent the expression of cholera toxin.

Definitions

[0065] The term "attenuated," when used with respect to bacteria, means that the bacteria have lost some or all of their ability to proliferate and/or cause disease or other adverse effect when they infect a host organism. For example, "attenuated" bacteria can be unable to replicate at all, or can be limited to one or a few rounds of replication, when present in a host organism in which a wild-type or other pathogenic version of the attenuated bacteria can replicate. Alternatively or additionally, "attenuated" bacteria might have one or more mutations in a gene or genes that are involved in the pathogenicity of the bacteria. Many genes, loci, or operons are known, mutations in which will result in attenuated bacteria. Examples of attenuated bacteria used as live vaccines include *S.* Typhi carrying a mutation in its galE or PhoP/Q genes, and *V. cholerae* carrying mutations in its ctxA gene.

[0066] A "host organism" is an animal that is a target of vaccination with the attenuated vaccines of the invention. Such host organisms have an immune system that is responsive to inoculation with an immunogen. Suitable host organisms include, for example,

humans, rodents, livestock, birds, and other animals in which it is desirable to vaccinate for either therapeutic or prophylactic purposes.

[0067] The terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression (*i.e.*, transcription and/or translation) of an operably linked coding sequence in a particular host organism. Expression vectors are exemplified by, but not limited to, plasmids, plasmid constructs, phagemids, shuttle vectors, cosmids, viral vectors, the chromosome, mitochondrial DNA, plastid DNA, and nucleic acid fragments, that may be used for expression of a desired sequence or sequences in a cell, such as a human cell, avian cell, fungal cell, protozoan cell, and/or insect cell. Nucleic acid sequences used for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome-binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. Flippase Recognition Target (FRT) sites and multiple cloning sites (MCS), also called polylinkers, may also be utilized. A MCS is a short DNA segment containing many (up to about 20) restriction sites that are typically unique, occurring only once within a given plasmid.

[0068] The term "transformation" as used herein, refers to any mechanism by which a DNA molecule (i.e., plasmid or PCR amplicon) may be incorporated into a host cell. A successful transformation results in the capability of the host cell to express any operative genes carried by the plasmid or incorporated into the chromosome. Transformations may result in genetically stable or transient gene expression. One example of a transient transformation comprises a plasmid vector within a cell, wherein the plasmid is not integrated into the host cell chromosome and is segregated from cells during cell division. Alternatively, a stable transformation comprises a plasmid within a cell, wherein the plasmid is integrated within the host cell genome or exists stably in the cytoplasm after many cell divisions.

[0069] The terms "subject", "patient", and "individual", as used herein, interchangeably refer to a multicellular animal (including mammals (*e.g.*, humans, non-Human primates, murines, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, ayes, *etc.*), avians (*e.g.*, chicken), amphibians (*e.g.* Xenopus), reptiles, and insects (*e.g.* Drosophila). "Animal" includes guinea pig, hamster, ferret, chinchilla, rabbit, mouse, and cotton rat.

[0070] As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST®, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, *et al.* 1990 *J Mol Biol* 215:403-410; Altschul, *et al.* 1997 *Methods in Enzymology*; Altschul, *et al.* 1997 *Nucleic Acids Res* 25:3389-3402; Baxevanis, *et al.* 1998 *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*, Wiley; and Misener, *et al.*, 1999 (eds.), *Bioinformatics Methods and Protocols* (Methods in Molecular Biology, Vol. 132), Humana Press; all of which are incorporated herein by reference. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology.

[0071] The phrases "homology" or "substantial homology", as used herein, refer to a comparison between amino acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be "substantially homologous" if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues with appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as "hydrophobic" or "hydrophilic" amino acids, and/or as having "polar" or "non-polar" side chains. Substitution of one amino acid for another of the same type may often be considered a "homologous" substitution.

[0072] In some embodiments, two sequences are considered to be substantially homologous if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are homologous over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more residues.

[0073] The phrases "identity" or "substantial identity", as used herein, refer to a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be "substantially identical" if they contain identical residues or bases in corresponding positions. Indeed, "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide

molecules. Calculation of the percent identity of two nucleic acid sequences can, for example, be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in stretches of one or both of a first and a second nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or substantially 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

[0074] In some embodiments, two sequences are considered to be substantially identical if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are identical over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more residues.

[0075] Reference herein to any numerical range expressly includes each numerical value (including fractional numbers and whole numbers) encompassed by that range. For example, but without limitation, reference herein to a range of 70 to 95% efficacy or to a pH range of 4.5 to 5.8 includes all whole numbers of and fractional numbers between the two. In a further illustration, reference herein to a range of "less than x" (wherein x is a specific number) includes whole numbers x-1, x-2, x-3, x-4, x-5, x-6, etc., and fractional numbers x-0.1, x-0.2, x-0.3, x-0.4, x-0.5, x-0.6, etc. In yet another illustration, reference herein to a range of from "x to y" (wherein x is a specific number, and y is a specific number) includes each whole number of x, x+1, x+2…to y-2, y-1, y, as well as each fractional number, such as x+0.1, x+0.2, x+0.3…to y-0.2, y-0.1. In another example, the term "at least 95%" includes each numerical value (including fractional numbers and whole numbers) from 95% to 100%, including, for example, 95%, 96%, 97%, 98%, 99% and 100%.

[0076] The terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," "immunologic," and "immunologically active", as used herein, refer to any substance that is capable of inducing a specific immune response (including eliciting a soluble antibody response) and/or cell-mediated immune response (including eliciting a cytotoxic T-lymphocyte (CTL) response).

[0077] An individual referred to as "suffering from" a disease, disorder, and/or condition (*e.g.*, influenza or typhoid fever or other bacterial infection) herein has been diagnosed with and/or displays one or more symptoms of a disease, disorder, and/or condition.

[0078] As used herein, the term "at risk" for disease (such as bacterial infection), refers to a subject (*e.g.*, a human) that is predisposed to contracting the disease and/or expressing one or more symptoms of the disease. Such subjects include those at risk for failing to elicit an immunogenic response to a vaccine against the disease. This predisposition may be genetic (*e.g.*, a particular genetic tendency to expressing one or more symptoms of the disease, such as heritable disorders, the presence of bacterial species blocking antibodies, the presence of reduced levels of bactericidal antibodies, *etc.*), or due to other factors (*e.g.*, immune suppressive conditions, environmental conditions, exposures to detrimental compounds, including immunogens, present in the environment, *etc.*). The term subject "at risk" includes subjects "suffering from disease," *i.e.*, a subject that is experiencing the disease. It is not intended that the present invention be limited to any particular signs or symptoms. Thus, it is intended that the present invention encompasses subjects that are experiencing any range of disease, from sub-clinical infection to full-blown disease, including wherein the subject exhibits at least one of the indicia (*e.g.*, signs and symptoms) associated with the disease.

[0079] The terms "treat," "treatment," or "treating", as used herein, refer to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition (*e.g.*, bacterial infection). Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition. In some embodiments, treatment may be administered to a subject who exhibits only early signs of the disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0080] As used herein, the terms "immunogenically effective amount," "immunologically effective amount", and "antigenically effective amount" refer to that

amount of a molecule that elicits and/or increases production of an immune response (including production of specific antibodies and/or induction of a TCL response) in a host upon vaccination. It is preferred, though not required, that the immunologically-effective (*i.e.*, immunogenically effective) amount is a "protective" amount. The terms "protective" and "therapeutic" amount of a composition or vaccine refer to an amount of the composition or vaccine that prevents, delays, reduces, palliates, ameliorates, stabilizes, and/or reverses disease (for example, bacterial infection) and/or one or more symptoms of disease.

[0081] As used herein, the term "vaccination" refers to the administration of a composition or vaccine intended to generate an immune response, for example, to a disease-causing agent. For the purposes of the present invention, vaccination can be administered before, during, and/or after exposure to a disease-causing agent, and, in certain embodiments, before, during, and/or shortly after exposure to the agent. In some embodiments, vaccination includes multiple administrations, appropriately spaced in time, of a vaccinating composition (vaccine).

[0082] The terms "comprises", "comprising", are intended to have the broad meaning ascribed to them in US Patent Law and can mean "includes", "including" and the like.

[0083] The term "vaccine," is used interchangeably herein with (as well as referred to as comprising) "immunoprotective composition" and, as used herein, refers to (comprising) an immunogen that, upon inoculation into a host organism, can induce complete or partial immunity to pathogenic agents or can reduce the effects of diseases associated with pathogenic agents.

[0084] The terms "nucleic acid molecule" or "oligonucleotide" or grammatical equivalents herein refer to at least two nucleotides covalently linked together. The term nucleic acid molecule may be used interchangeably with gene, cDNA, and mRNA encoded by a gene. A nucleic acid molecule of the present invention is preferably single-stranded or double-stranded and will generally contain phosphodiester bonds, although, in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al. (1993) Tetrahedron 49(10):1925) and references therein; Letsinger (1970) J. Org. Chem. 35:3800; Sprinzl et al. (1977) Eur. J. Biochem. 81:579; Letsinger et al. (1986) Nucl. Acids Res. 14:3487; Sawai et al. (1984) Chem. Lett. 805, Letsinger et al. (1988) J. Am. Chem. Soc. 110:4470; and Pauwels et al. (1986) Chemica Scripta 26:141 9), phosphorothioate (Mag et al. (1991) Nucleic Acids Res. 19:1437; and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu et al. (1989) J. Am. Chem. Soc. 111:2321, O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and

Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm (1992) J. Am. Chem. Soc. 114:1895; Meier et al. (1992) Chem. Int. Ed. Engl. 31:1008; Nielsen (1993) Nature, 365:566; Carlsson et al. (1996) Nature 380:207). Other analog nucleic acids include those with positive backbones (Denpcy et al. (1995) Proc. Natl. Acad. Sci. USA 92:6097; non-ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Angew. (1991) Chem. Intl. Ed. English 30:423; Letsinger et al. (1988) J. Am. Chem. Soc. 110:4470; Letsinger et al. (1994) Nucleoside & Nucleotide 13:1597; Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook; Mesmaeker et al. (1994), Bioorganic & Medicinal Chem. Lett. 4:395; Jeffs et al. (1994) J. Biomolecular NMR 34:17; Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, Carbohydrate Modifications in Antisense Research, Ed. Y. S. Sanghui and P. Dan Cook. Nucleic acid molecules containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al. (1995), Chem. Soc. Rev. pp 169-176). Several nucleic acid analogs are described in Rawls, C & E News Jun. 2, 1997 page 35. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments.

[0085] An "exogenous DNA segment", "heterologous sequence", or a "heterologous nucleic acid", as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell, but has been modified. Thus, the terms refer to a DNA segment which is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

[0086] The term "gene" is used broadly to refer to any segment of DNA associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include non-expressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information and may include sequences designed to have desired parameters.

[0087] The terms "polypeptide," "peptide", and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms additionally apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[0088] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0089] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-RJB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0090] The terms “variants” and "conservatively modified variants", as used herein, apply to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, variants imply functional variants, and conservatively modified variants refer to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG,

which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0091] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions, or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alter, add, or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in the encoded sequence are "conservatively modified variants," where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0092] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (1), Leucine (L), Methionine (M), Valine (V);

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

7) Serine (S), Threonine (T); and

8) Cysteine (C), Methionine (M)

[0093] The term "isolated", when applied to a nucleic acid molecule or gene or protein, denotes that the nucleic acid molecule or gene or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state, although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is considered substantially purified. In particular, an isolated gene is separated from open reading frames which flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid molecule or gene or protein gives rise to essentially one band in an electrophoretic gel.

Particularly, it means that the nucleic acid molecule or gene or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

[0094] The term "naturally-occurring" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, an organism, or a polypeptide or polynucleotide sequence that is present in an organism (including viruses, bacteria, protozoa, insects, plants or mammalian tissue) that can be isolated from a source in nature and that has not been intentionally modified by man in the laboratory is naturally-occurring.

[0095] A nucleic acid sequence or gene is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence or gene. For instance, a promoter or enhancer is operably linked to a coding sequence if it increases the transcription of the coding sequence. Operably linked means that the sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

[0096] The term "recombinant" when used with reference to bacteria indicates that the host bacteria contains a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Heterologous nucleic acids can, for example, integrate into the host bacteria chromosome and be expressed from host or heterologous promoters. Alternatively, heterologous nucleic acids can be expressed from an autonomously replicating plasmid. Recombinant bacteria can contain genes that are not found within the native (non-recombinant) form of the bacteria. Recombinant bacteria can also contain genes found in the native form of the bacteria, wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses bacteria that contain a nucleic acid endogenous to the bacteria that has been modified without removing the nucleic acid from the bacteria; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

[0097] A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes may include promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide or series of peptides) and a

promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein from the host cell. Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette. The recombinant expression cassette may be located on an autonomously replicating plasmid or may be integrated into the host genome.

[0098] The term "selectable marker" refers to a nucleotide sequence that encodes a protein that confers either a positive or negative selective advantage to bacteria expressing that marker. For example, an expression cassette comprising a selectable marker could comprise the aspartate β-semialdehyde dehydrogenase (asd) gene operably linked to a promoter. A recombinant plasmid capable of expressing asd could complement the asd phenotype of asd deletion mutants. Bacteria lacking asd would not be able to synthesize diaminopimelic acid, an essential element of the peptidoglycan of the bacterial cell wall and would die. Examples of other selectable markers useful in bacteria include SacB, aroA, and heavy metal ion resistance genes.

[0099] The term "protective immunity" means that a vaccine or immunization schedule that is administered to a mammal induces an immune response that prevents, retards the development of, or reduces the severity of a disease or infection that is caused by a bacterial species, or diminishes or altogether eliminates the symptoms of the disease or infection.

[00100] The phrase "sufficient to invoke an immunoprotective response" means that there is a detectable difference between an immune response indicator measured before and after administration of a particular antigen preparation. Immune response indicators include but are not limited to: antibody titer or specificity, as detected by an assay such as enzyme-linked immunoassay (ELISA), bactericidal assay, flow cytometry, immunoprecipitation, Ouchter-Lowny immunodiffusion; binding detection assays of, for example, spot, Western blot or antigen arrays; cytotoxicity assays, etc.

[00101] A "surface antigen" is an antigen that is present in a surface structure of a bacteria for example, is capable of generating an immunoprotective response when expressed by a recombinant bacteria and presented to a host organism in an immunoprotective composition.

[00102] Unless otherwise indicated, all numbers expressing quantities of ingredients, dimensions reaction conditions and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about".

[00103] In this application and the claims, the use of the singular includes the plural unless specifically stated otherwise. In addition, use of "or" means "and/or" unless stated otherwise. Moreover, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one unit unless specifically stated otherwise.

Additional embodiments of the invention

[00104] In one embodiment, an immunoprotective composition according to the invention comprises a prokaryotic microorganism. In another embodiment, the prokaryotic microorganism is a bacterial species. Preferably, the prokaryotic microorganism is an attenuated strain of Salmonella. However, other prokaryotic microorganisms, such as attenuated strains of *Escherichia coli*, Shigella, Yersinia, Lactobacillus, Mycobacteria, Listeria or Vibrio are likewise contemplated for the invention. Examples of suitable strains of microorganisms include, but are not limited to, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella dublin*, *Salmonella enteritidis*, *Escherichia coli*, *Shigella flexnieri*, *Shigella sonnei*, *Vibrio cholerae* (Yamamoto, et al. (2009) Gene 438:57-64), *Pseudomonas aeroginosa* (Lesic, et al. (2008) BMC Mol Biol 9:20), *Yersinia pestis* (Sun, et al. (2008) Applied and Env Microbiol 74:4241-4245), and *Mycobacterium bovis* (BCG). Of note, lambda red does not work in Mycobacteria, but another phage (for example, Che9c gp61) has been used for recombineering in Mycobacteria (van Kessel, et al. (2008) Methods mol biol 435:203-215).

[00105] In one embodiment, the prokaryotic microorganism is *Salmonella typhi* Ty21a. VIVOTIF® Typhoid Vaccine Live Oral Ty21a is a live attenuated vaccine intended for oral administration. The vaccine contains the attenuated strain *Salmonella Typhi* Ty21a. (Germanier *et al.* 1975 *J Infect Dis* 131:553-558; US 3,856,935).

[00106] In a further aspect, the present invention provides immunogenic compositions comprising one or more of above attenuated prokaryotic microorganisms, optionally in combination with a pharmaceutically or physiologically acceptable carrier. Preferably, the composition is a vaccine, especially a vaccine for mucosal immunization, *e.g.*, for administration via the oral, rectal, nasal, vaginal or genital routes.

[00107] In a further aspect, the present invention provides an attenuated strain of a prokaryotic microorganism described above for use as a medicament, especially as a vaccine.

[00108] In a further aspect, the present invention provides the use of an attenuated strain of a prokaryotic microorganism comprising a glutamic acid decarboxylase (gad) gene region inserted into a chromosome of the prokaryotic microorganism, wherein the Gad proteins are produced in the microorganism, in the preparation of a medicament for the prophylactic or therapeutic treatment of one or several different types of bacterial infection.

[00109] Generally, the microorganisms expressing Gads according to the present invention are provided in an isolated and/or purified form, *i.e.*, substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients. A composition according to the present invention may include in addition to the microorganisms expressing Gads as disclosed, one or more other active ingredients for therapeutic or prophylactic use (*e.g.*, other antigens) and an adjuvant.

[00110] The compositions of the present invention are preferably given to an individual in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of who/what is being treated. Prescription of treatment, *e.g.*, final decisions on acceptable dosage etc., will be dictated by Vaccine Regulatory Authorities, after review of safety and efficacy data following human immunizations.

[00111] A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

[00112] Compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient (immunogen) and/or acid resistance (*gad*) gene(s), a pharmaceutically or physiologically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of, but may aid the active ingredient. In certain embodiments, arabinose and/or glutamate are added to the composition or vaccine. These additional components may be needed in the oral cavity, where they are not present. The precise nature of the carrier or other material will depend on the route of administration.

[00113] The invention further relates to the identification and sequencing of three *gad* genes from *Shigella flexneri* 2a str. 2457T complete (available, for example, via GenBank as "complete genome", 4,599,354 bp); Wei, *et al.* 2003 *Infect Immun* 71 (5):2775-2786: gadA (SEQ ID NO:3), gadB (SEQ ID NO:4), and gadC/xasA (SEQ ID NO:5) acid sensitivity protein, antiporter. Of note, the sequence identity between gadA and gadB at the DNA level is 96%. These sequences are incorporated herein in their entirety.

[00114] These genes may be present in whole or in part in the immunogenic compositions described herein.

[00115] Accordingly, the present invention relates to immunogenic compositions further characterized by the presence of heterologous genes or a set of heterologous genes coding for the Gads.

[00116] In one embodiment of the immunogenic compositions, the heterologous gene(s) is (are) stably integrated into the chromosome of said strain at a defined integration site, which is to be nonessential for growth, for inducing a protective immune response (and/or acid resistance) by the carrier strain.

[00117] The *gad* genes may, individually or together or both, be under the control of the cognate promoter or other non-cognate promoters. The gad genes may be physically together in one operon or separated on the chromosome and present on separate DNA regions under the control of different promoters. The genes may vary in gene order when placed next to one another, as long as they are biologically functional.

[00118] Alternatively, the above immunogenic compositions contain *gad* genes and/or additional gene(s) integrated in tandem into a single chromosomal site or located at separate chromosomal sites.

[00119] The invention also relates to a live vaccine comprising immunogenic compositions according to the invention and, optionally, a pharmaceutically or physiologically acceptable carrier and/or a buffer for neutralizing gastric acidity and/or a system for delivering the vaccine in a viable state to the intestinal tract.

[00120] The vaccine comprises an immunoprotective or immunotherapeutic and non-toxic amount of the immunogenic composition. Suitable dosage amounts can be determined by the person skilled in the art and are typically 10^7 to 10^{10} bacteria given orally.

[00121] Pharmaceutically and physiologically acceptable carriers, suitable neutralizing buffers, and suitable delivering systems can be selected by the person skilled in the art.

[00122] The mode of administration of the vaccines of the present invention may be any suitable route which delivers an immunoprotective or immunotherapeutic amount of the vaccine to the subject. However, the vaccine is preferably administered orally.

[00123] In certain embodiments, the invention includes naturally occurring and/or unnaturally occurring polynucleotides and polypeptide products thereof. Naturally occurring Gads include distinct gene and polypeptide species as well as corresponding species homologs expressed in organisms other than *Shigella* or *Salmonella* strains. Non-naturally occurring Gads include variants of the naturally occurring products such as analogs and gads that include covalent modifications.

[00124] "Synthesized," as used herein and is understood in the art, refers to purely chemical, as opposed to enzymatic, methods for producing polynucleotides. "Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means.

[00125] Autonomously replicating recombinant expression constructions such as plasmid and viral DNA vectors incorporating *gad* gene sequences are also provided. Expression constructs wherein Gad polypeptide-encoding polynucleotides are operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided. The *gad* genes may be cloned by PCR, using genomic DNA from any *Shigella* species or certain *E. coli* strains as the template. For ease of inserting the gene into expression vectors, PCR primers are chosen so that the PCR-amplified gene(s) has a restriction enzyme site at the 5' end preceding the initiation codon ATG, and a restriction enzyme site at the 3' end after the termination codon TAG, TGA or TAA. If desirable, the codons in the gene(s) are changed, without changing the amino acids, according to *E. coli* codon preference described by Grosjean, *et al.* 1982 *Gene* 18:199-209; and Konigsberg, *et al.* 1983 *PNAS USA* 80:687-691. Optimization of codon usage may lead to an increase in the expression of the gene product when produced in *E. coli* or *Salmonella*. If a protein gene product is to be produced extracellularly, either in the periplasm of *E. coli* or other bacteria, or into the cell culture medium, the gene is cloned into an expression vector and linked to a signal sequence.

[00126] In one embodiment, the attenuated strain of the prokaryotic microorganism undergoes recombineering, such that a large antigenic region is integrated into the chromosome of the microorganism.

[00127] According to another aspect of the invention, host cells are provided, including prokaryotic and eukaryotic cells, either stably or transiently transformed, transfected, or electroporated with polynucleotide sequences of the invention in a manner which permits expression of Gad polypeptides of the invention. Potential expression systems of the invention include bacterial, yeast, fungal, viral, parasitic, invertebrate, and mammalian cells systems. Gads produced in humans/animals may result in death of cancer cells or other beneficial effects, particularly when delivered by attenuated *Salmonella*. Host cells of the invention are conspicuously useful in methods for large scale production of Gad polypeptides, wherein the cells are grown in a suitable culture medium, and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by, for example, immunoaffinity purification or any of the multitude of purification techniques well known and routinely practiced in the art. Any suitable host cell may be used for expression of the gene product, such as *E. coli*, other bacteria, including *P. multocida*, *Bacillus* and *S. aureus*, yeast, including *Pichia pastoris* and *Saccharomyces cerevisiae*, insect cells, or mammalian cells, including CHO cells, utilizing suitable vectors known in the art. Proteins may be produced directly or fused to a peptide or polypeptide, and either intracellularly or extracellularly by secretion into the periplasmic space of a bacterial cell or into the cell culture medium. Secretion of a protein requires a signal peptide (also known as pre-sequence); a number of signal sequences from prokaryotes and eukaryotes are known to function for the secretion of recombinant proteins. During the protein secretion process, the signal peptide is removed by signal peptidase to yield the mature protein.

[00128] To simplify the protein purification process, a purification tag may be added either at the 5' or 3' end of the gene coding sequence. Commonly used purification tags include a stretch of six histidine residues (US Patent Nos. 5,284,933 and 5,310,663), a streptavidin¬ affinity tag described by Schmidt et al. (1993) Protein Eng., 6:109-122, a FLAG peptide (Hopp et al. (1988) Biotechnology, 6:1205-1210), glutathione 5-transferase (Smith et al. (1988) Gene, 67:31-40), and thioredoxin (LaVallie et at. (1993) Bio/Technology, 11:187-193). To remove these peptide or polypeptides, a proteolytic cleavage recognition site may be inserted at the fusion junction. Commonly used proteases are factor Xa, thrombin, and enterokinase.

[00129] In one embodiment, the invention employs purified and isolated *Shigella* species Gad polypeptides as described above. Presently preferred are polypeptides comprising the amino acid sequences encoded by any one of the polynucleotides set out in SEQ ID NOs:3, 4, and/or 5, and functional variants thereof. Functional variants refer to

mutated Gad DNA sequences, made by existing genetic/synthetic procedures, that result in proteins/peptides that retain sufficient function (here, decarboxylase or antiporter activities) to be useful. In certain embodiments, the invention utilizes gad polypeptides encoded by a DNA selected from the group consisting of:

[00130] a) the DNA sequence set out in SEQ ID NOs:3, 4, and/or 5 and functional variants thereof;

[00131] b) DNA molecules encoding *Shigella sonnei* Gad polypeptides encoded by any one of SEQ ID NO:3, 4, and/or 5 and functional variants thereof; and

[00132] c) a DNA molecule encoding the *gad* gene products that hybridizes under moderately stringent conditions to the DNA of (a) or (b). Moderately stringent hybridization conditions are well-known to the ordinarily skilled artisan.

[00133] The invention also embraces polypeptides that have at least about 99%, at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, and at least about 50% identity and/or homology to the preferred polypeptides of the invention.

[00134] Conservative substitutions can be defined as set out below in Tables B and C, below.

[00135] TABLE B. Conservative Substitutions I

SIDE CHAIN	CHARACTERISTIC	AMINO ACID
Aliphatic	Non-polar	G, A, P, I, L, V
	Polar-uncharged	C, S, T, M, N, Q
	Polar-charged	D, E, K, R
Aromatic		H, F, W, Y
Other		N, Q, D, E

[00136] Polypeptides of the invention may be isolated from natural bacterial cell sources or may be chemically synthesized but are preferably produced by recombinant procedures involving host cells of the invention. Gad gene products of the invention may be full-length polypeptides, biologically active fragments, or variants thereof which retain specific biological activity. The biological activity is, in one embodiment, acid resistance of the bacterial species (or microorganism species). Such functional variants may comprise gad polypeptide analogs wherein one or more of the specified (*i.e.*, naturally encoded) amino acids is deleted or replaced or wherein one or more non-specified amino acids are added: (1) without loss of the biological activities. Deletion variants contemplated also include

fragments lacking portions of the polypeptide not essential for biological activity, and insertion variants include fusion polypeptides in which the wild-type polypeptide or fragment thereof have been fused to another polypeptide.

[00137] Variant Gad polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention. Conservative substitutions are recognized in the art to classify amino acids according to their related physical properties and can be defined as set out in Table B, above, and/or C, below.

[00138] TABLE B. Conservative Substitutions II

SIDE CHAIN CHARACTERISTIC AMINO ACID

Non-polar (hydrophobic)

A. Aliphatic: A, L, I, V, P

B. Aromatic: F, W

C. Sulfur-containing: M

D. Borderline: G

Uncharged-polar

A. Hydroxyl: S, T, Y

B. Amides: N, Q

C. Sulfhydryl: C

D. Borderline: G

Positively Charged (Basic): K, R, H

Negatively Charged (Acidic): D, E

[00139] The invention also embraces variant polypeptides having additional amino acid residues which result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as fusion protein with glutathione-S-transferase (GST) provide the desired polypeptide having an additional glycine residue at position -1 following cleavage of the GST component from the desired polypeptide. Variants which result from expression using other vector systems are also contemplated.

Treatment/Therapy

[00140] In certain embodiments, the present invention provides compositions (including vaccines) and methods to treat (e.g., alleviate, ameliorate, relieve, delay onset of,

inhibit progression of, reduce severity of, and/or reduce incidence of one or more symptoms or features of) and/or prevent bacterial infection.

[00141] In some embodiments, methods of vaccination and/or treatment (such as those described in the sections below) involve stratification of a patient population based on prior exposure to bacterial strains. Such methods involve steps of determining whether a patient has been previously exposed to one or more of the bacterial strains. In some embodiments, if it is determined that a patient has been previously been exposed to one or more of the bacterial strains, that patient may receive less concentrated, less potent, and/or less frequent doses of the inventive vaccine or composition. If it is determined that a patient has not been previously been exposed to one or more of the bacterial strains, that patient may receive more concentrated, more potent, and/or more frequent doses of the inventive vaccine or composition.

[00142] In one embodiment, the vaccine or composition of the invention treats more than one bacterial infection, *i.e.*, infection with more than one bacterial species. It can, in such an embodiment, be deemed a "multifunctional" vaccine (or strain or composition – either of the latter two are contemplated in the continued description, below). A multifunctional vaccine according to the invention may also be useful for treating and/or preventing simultaneously a number of different disorders in a subject. Accordingly, the present invention further provides, in an additional embodiment, a method for treating and/or preventing more than one disorder in a subject, by administering to the subject a multifunctional vaccine according to the invention.

[00143] Exemplary disorders which potentially may be treated and/or prevented by a multifunctional vaccine according to the invention include, without limitation, multiple bacterial species infections, burns, infections, neoplasia, or radiation injuries. "Neoplasia" refers to the uncontrolled and progressive multiplication of tumour cells, under conditions that would not elicit, or would cause cessation of, multiplication of normal cells. Neoplasia results in a "neoplasm", which is defined herein to mean any new and abnormal growth, particularly a new growth of tissue, in which the growth of cells is uncontrolled and progressive. Thus, neoplasia includes "cancer", which herein refers to a proliferation of tumour cells having the unique trait of loss of normal controls, resulting in unregulated growth, lack of differentiation, local tissue invasion, and/or metastasis. This would, for example, be relevant if the tumor was located in the intestine, and the Ty21a(Gad)-expressing anti-tumor drugs were being given orally. However, certain attenuated *Salmonella* strains

expressing Gad genes can be administered intravenously and specifically target tumor cells at many sites within the host.

Administration

[00144] Compositions and vaccines may be administered using any amount and any route of administration effective for treatment and/or vaccination. The exact amount required may vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the infection, the particular composition, its mode of administration, its mode of activity, and the like. Compositions are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions and vaccine of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject or organism will depend upon a variety of factors including the disorder being treated and/or vaccinated and the severity of the disorder; the activity of the specific vaccine composition employed; the half-life of the composition after administration; the age, body weight, general health, sex, and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors, well known in the medical arts.

[00145] Compositions (and vaccines) may be administered by any route, including oral (PO), parenteral, intravenous (IV), intramuscular (IM), intra-arterial, intramedullary, intrathecal, subcutaneous (SQ), intraventricular, transdermal, interdermal, intradermal, rectal (PR), vaginal, intraperitoneal (IP), intragastric (IG), topical or transcutaneous (*e.g.*, by powders, ointments, creams, gels, lotions, and/or drops), mucosal, intranasal, buccal, enteral, vitreal, sublingual; by intratracheal instillation, bronchial instillation, and/or inhalation; as an oral spray, nasal spray, and/or aerosol, and/or through a portal vein catheter. However, in a preferred embodiment, the disclosed compositions and vaccines are administered orally.

[00146] For oral administration, the composition (including vaccine) may be presented as capsules, tablets, dissolvable membranes, powders, granules, or as a suspension. The composition may have conventional additives, such as lactose, mannitol, corn starch, or potato starch. The composition also may be presented with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch, or gelatins. Additionally, the composition may be presented with disintegrators, such as corn starch, potato starch, or sodium carboxymethylcellulose. The composition may be further presented with dibasic calcium

phosphate anhydrous or sodium starch glycolate. Finally, the composition may be presented with lubricants, such as talc or magnesium stearate.

[00147] Solid dosage forms for oral administration include capsules, tablets, pills, powders, dissolvable membranes/wafers, and granules. In such solid dosage forms, the active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.*, starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.*, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.*, glycerol), disintegrating agents (*e.g.*, agar, calcium carbonate, potato starch, tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.*, paraffin), absorption accelerators (*e.g.*, quaternary ammonium compounds), wetting agents (*e.g.*, cetyl alcohol and glycerol monostearate), absorbents (*e.g.*, kaolin and bentonite clay), and lubricants (*e.g.*, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), taste/olfactory components, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

[00148] Sublingual vaccination has been used to deliver drugs and small molecules to the bloodstream. Sublingual vaccination constitutes a form of the vaccine (solution, suspension, wafer, film strip, etc.) being placed under the tongue. Due to the presence of high density blood vessels in the mucous membranes, the immune system cells capture the vaccine and move the vaccine through the body without significant degradation. Sublingual vaccination appears to disseminate immunity to a broader range of organs than the classical routes of injecting or ingesting vaccines. Sublingual vaccination likewise eliminates the need for the use of needle and the risks associated with the needle injection method.

[00149] An objective of the instant study was to develop a wafer formulation that is robust and can disintegrate in water (surrogate for saliva) in a reasonable amount of time. The robustness of a wafer is usually proportional to the amount of excipient added.

[00150] Orally disintegrating tablets (ODTs) offer improved patient compliance, as they enable oral administration without water or chewing. The US FDA defines an ODT as "a solid dosage form containing medicinal substances which disintegrates rapidly, usually within a matter of seconds, when placed upon the tongue." The 2008 FDA guidance recommends a disintegration time of 30 seconds or less based on US Pharmacopeia disintegration test method and maximum tablet weight of 500 mg. ODTs are preferred by multiple patients groups with swallowing difficulties, including geriatrics, pediatrics, dysphagic, and bed ridden, and, most significantly, in developing countries. ODTs also offer

potential for product line extension for first-to-market product and marketing differentiation for Over the Counter Products (OTCs).

[00151] Compression and lyophilization remain the two most popular industrial approaches to manufacture orally disintegrating tablets (ODTs). The compressed ODT involves conventional tableting with rapid disintegration using super-disintegrants in combination with lower compression forces and/or the use of water-soluble excipients. Direct compression is often the technique of choice. Some of the patented compressed ODT technologies include Flashtab, Advatab, Orasolv, Durasolv, Wowtab, and Ziplets. The lyophilized ODT employs the process of lyophilization in which solvent is removed from a frozen drug solution or suspension containing structure forming excipients. The lyophilization manufacturing process produces wafer with greater porosity, allowing for shorter disintegration times than compressed ODTs. The patented lyophilized ODT technologies include Zydis, Lyoc, and Quicksolv. Other techniques to manufacture ODTs include spray drying or foam drying, molding, thin films, melt granulation, extrusion, and sugar floss.

[00152] Lyoc technology is an oral solid porous dosage form that immediately dissolves in the mouth without the need for water. Lyoc utilizes a manufacturing process in the absence of organic solvents that includes the steps of: preparation of a suspension, solution, or emulsion containing active ingredients; distribution of this liquid homogenous preparation in preformed blisters; very low temperature freezing (preferably below -40°C), sublimation or water elimination, with the finished product being a porous solid capable of very rapid disintegration, and sealing the blister with top foil.

[00153] Dry powder vaccine formulations are also prepared using the Sievers et al patented Carbon Dioxide Assisted Nebulization with a Bubble Dryer (CAN- BD) drying process. Desired wafer properties were tested, *i.e.* binding capabilities, target dissociation time.

Vaccines

[00154] A "vaccine" is a composition that induces an immune response in the recipient or host of the vaccine. The vaccine can induce protection against infection upon subsequent challenge with a bacterial species (or other microorganism). Protection refers to resistance (e.g., partial resistance) to persistent infection of a host animal with at least one bacterial species (or other microorganism). Neutralizing antibodies generated in the vaccinated host can provide this protection. In other situations, CTL responses can provide this protection. In

some situations, both neutralizing antibodies and cell-mediated immune (*e.g.*, CTL) responses provide this protection.

[00155] Vaccines are useful in preventing or reducing infection or disease by inducing immune responses, to an antigen or antigens, in an individual. For example, vaccines can be used prophylactically in naive individuals, or therapeutically in individuals already infected with at least one bacterial species (or other microorganism).

[00156] Protective responses can be evaluated by a variety of methods. For example, either the generation of neutralizing antibodies against bacterial (or other microorganism) proteins, and/or the generation of a cell-mediated immune response against such proteins can indicate a protective response. Protective responses also include those responses that result in lower number of bacteria colonized in a vaccinated host animal exposed to a given inoculum (of bacteria or other microorganism) as compared to a host animal exposed to the same inoculum, but that has not been administered the vaccine.

[00157] Vaccines according to the invention may, in one embodiment, contain an adjuvant. The term "adjuvant", as used herein, refers to any compound which, when injected together with an antigen, non-specifically enhances the immune response to that antigen. Exemplary adjuvants include Complete Freund's Adjuvant, Incomplete Freund's Adjuvant, Gerbu adjuvant (GMDP; C.C. Biotech Corp.), RIBI fowl adjuvant (MPL; RIBI Immunochemical Research, Inc.), potassium alum, aluminum phosphate, aluminum hydroxide, QS21 (Cambridge Biotech), TITERMAX® adjuvant (CytRx Corporation), and QUIL A® adjuvant. Other compounds that may have adjuvant properties include binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, PRIMOGEL®, corn starch and the like; lubricants such as magnesium stearate or STEROTEX®; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin, a flavoring agent such as peppermint, methyl salicylate or orange flavoring, and a coloring agent.

[00158] Furthermore, a useful compendium of many adjuvants is prepared by the National Institutes of Health and can be found on the internet (www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf). Hundreds of different adjuvants are known in the art and could be employed in the practice of the present invention. Exemplary adjuvants that can be utilized in accordance with the invention include, but are not limited to, cytokines, aluminum salts (*e.g.,* aluminum hydroxide, aluminum phosphate, etc.), gel-type adjuvants (*e.g.*, calcium phosphate, etc.); microbial adjuvants (*e.g.*, immunomodulatory DNA

sequences that include CpG motifs; endotoxins such as monophosphoryl lipid A); exotoxins such as cholera toxin, *E. coli* heat labile toxin, and pertussis toxin; muramyl dipeptide, etc.); oil-emulsion and emulsifier-based adjuvants (*e.g.*, Freund's Adjuvant, MF59 [Novartis], SAF, etc.); particulate adjuvants (*e.g.*, liposomes, biodegradable microspheres, etc.); synthetic adjuvants (*e.g.*, nonionic block copolymers, muramyl peptide analogues, polyphosphazene, synthetic polynucleotides, etc.); and/or combinations thereof. Other exemplary adjuvants include some polymers (e.g., polyphosphazenes), Q57, saponins (*e.g.*, QS21), squalene, tetrachlorodecaoxide, CPG 7909, poly[di(carboxylatophenoxy)phosphazene] (PCCP), interferon-gamma, block copolymer P1205 (CRL1005), interleukin-2 (IL-2), polymethyl methacrylate (PMMA), etc. In one embodiment of the instant invention, the carrier bacterium (*e.g. Salmonella* Typhi Ty21a) itself serves as an adjuvant for expressed foreign antigens such as *Shigella* O antigens or anthrax protective antigen.

[00159] Vaccines according to the invention may, in another embodiment, be formulated using a diluent. Exemplary "diluents" include water, physiological saline solution, human serum albumin, oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol, antioxidants such as ascorbic acid or sodium bisulphite, chelating agents such as ethylene diamine-tetra-acetic acid, buffers such as acetates, citrates or phosphates and agents for adjusting the osmolarity, such as sodium chloride or dextrose. Exemplary "carriers" include liquid carriers (such as water, saline, culture medium, saline, aqueous dextrose, and glycols) and solid carriers (such as carbohydrates exemplified by starch, glucose, lactose, sucrose, and dextrans, anti-oxidants exemplified by ascorbic acid and glutathione, and hydrolyzed proteins.

[00160] Vaccines according to the invention may, in still another embodiment, contain an excipient. The term "excipient" refers herein to any inert substance (e.g., gum arabic, syrup, lanolin, starch, etc.) that forms a vehicle for delivery of an antigen. The term excipient includes substances that, in the presence of sufficient liquid, impart to a composition the adhesive quality needed for the preparation of pills or tablets.

[00161] As mentioned above, in some embodiments, interfering agents and/or binding agents in accordance with the invention may be utilized for prophylactic applications. In some embodiments, prophylactic applications involve systems and methods for preventing, inhibiting progression of, and/or delaying the onset of bacterial infection. In some embodiments, interfering agents may be utilized for passive immunization (*i.e.*, immunization wherein antibodies are administered to a subject). In some embodiments, vaccines for passive immunization may comprise antibody interfering agents, such as those described herein. In

some embodiments, passive immunization occurs when antibodies are transferred from mother to fetus during pregnancy. In some embodiments, antibodies are administered directly to an individual (*e.g.*, by injection, orally, *etc.*). Of note, it is possible to immunize a person and use their antibodies for passive protection in another individual. For example, Ty21a is contraindicated for use in pregnant women (*i.e.*, they are immunocompromised, and the attenuated *Salmonella* could potentially cause illness). However, it may be appropriate to passively transfer protective antibodies to a pregnant mother, instead of leaving them susceptible to deadly diseases.

[00162] The invention provides, in one embodiment, vaccines for active immunization (*i.e.*, immunization wherein microbes, proteins, peptides, epitopes, mimotopes, etc. are administered to a subject). In some embodiments, the vaccines may comprise one or more interfering agents and/or binding agents, as described herein.

[00163] In one embodiment, a composition is provided including interfering agents and/or binding agents, as described for vaccines, above. For example, in some embodiments, interfering agent and/or binding agent polypeptides, nucleic acids encoding such polypeptides, characteristic or biologically active fragments of such polypeptides or nucleic acids, antibodies that bind to and/or compete with such polypeptides or fragments, small molecules that interact with or compete with such polypeptides or with glycans that bind to them, etc. are included in compositions. In some embodiments, interfering agents and/or binding agents that are not polypeptides, *e.g.*, that are small molecules, umbrella topology glycans and mimics thereof, carbohydrates, aptamers, polymers, nucleic acids, etc., are included in the compositions. One could, in specific embodiments, envision bacterially delivered therapies for cancer or other maladies, in which the therapeutic nucleic acids or proteins are encoded in the chromosome of the carrier bacterial vaccine/therapeutic strain

[00164] The invention encompasses treatment and/or prophylaxis of bacterial (or other microorganism) infections by administration of compositions according to the invention. In some embodiments, such compositions are administered to a subject suffering from or susceptible to a bacterial infection. In some embodiments, a subject is considered to be suffering from a bacterial infection if the subject is displaying one or more symptoms commonly associated with the bacterial infection. In some embodiments, the subject is known or believed to have been exposed to the at least one bacterial species (or other microorganism). In some embodiments, a subject is considered to be susceptible to a bacterial infection if the subject is known or believed to have been exposed to the bacterial species. In some embodiments, a subject is known or believed to have been exposed to the bacterial

species if the subject has been in contact with other individuals known or suspected to have been infected with the same, and/or if the subject is or has been present in a location in which the bacterial infection is known or thought to be prevalent. Compositions provided herein may be administered prior to or after development of one or more symptoms of bacterial (or other microorganism) infection.

[00165] In general, a composition may include a "therapeutic agent" (the attenuated bacteria or attenuated bacteria expressing one or more foreign antigens, for example) and the three acid resistance (Gad) genes described herein, in addition to one or more inactive, agents such as a sterile, biocompatible pharmaceutical carrier including, but not limited to, sterile water, saline, buffered saline, or dextrose solution. Alternatively or additionally, a composition may comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, disintegrating agents, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, buffering agents, solid binders, granulating agents, lubricants, coloring agents, sweetening agents, flavoring agents, perfuming agents, and the like, as suited to the particular dosage form desired. Remington's The Science and Practice of Pharmacy, 21st Ed., A. R. Gennaro, (Lippincott, Williams & Wilkins, Baltimore, Md., 2006; incorporated herein by reference) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

Combinations

[00166] Compositions and vaccines according to the invention can be administered to a subject either alone or in combination with one or more other therapeutic agents including, but not limited to, vaccines and/or antibodies. By "in combination with," it is not intended to imply that the agents must be administered at the same time or formulated for delivery together, although these methods of delivery are within the scope of the invention. Compositions and vaccines according to the invention can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. It will be appreciated that therapeutically active agents utilized in combination may be administered together in a single composition or administered separately in different

compositions. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent.

[00167] In general, each agent (in this context, one of the "agents" is a composition or vaccine according to the invention) will be administered at a dose and on a time schedule determined for that agent. Additionally, the invention encompasses the delivery of the compositions in combination with agents that may improve their bioavailability, reduce or modify their metabolism, inhibit their excretion, or modify their distribution within the body. Although the compositions (including vaccines) according to the invention can be used for treatment and/or vaccination of any subject, they are preferably used in the treatment and/or vaccination of humans. Inclusion of the *gad* genes in vectors designed for oral use in animals would be appropriately used in those targeted animal species.

[00168] The particular combination of therapies (*e.g.*, therapeutics or procedures) to employ in a combination regimen will take into account compatibility of the desired therapeutics and/or procedures and the desired therapeutic effect to be achieved. It will be appreciated that the therapies employed may achieve a desired effect for the same purpose (for example, an agent useful for treating, preventing, and/or delaying the onset of a bacterial (or other microorganism) infection may be administered concurrently with another agent useful for treating, preventing, and/or delaying the onset of the bacterial infection), or they may achieve different effects (e.g., prevention of severe illness or control of adverse effects).

[00169] A composition (including vaccine) according to the invention may be administered to a subject who has a disorder, either alone or in combination with one or more drugs used to treat that disorder. For example, where the subject has neoplasia, the composition may be administered to a subject in combination with at least one antineoplastic drug. Examples of antineoplastic drugs with which the composition may be combined include, without limitation, carboplatin, cyclophosphamide, doxorubicin, etoposide, and vincristine. Additionally, when administered to a subject who suffers from neoplasia, the composition may be combined with other neoplastic therapies, including, without limitation, surgical therapies, radiotherapies, gene therapies, and immunotherapies.

[00170] In general, it is expected that agents utilized in combination with be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually.

Dosages

[00171] The dosage of a vaccine (or other composition) according to the invention can be determined by, for example, first identifying doses effective to elicit a prophylactic and/or therapeutic immune response. This may be accomplished by measuring the serum titer of bacterium-specific immunoglobulins and/or by measuring the inhibitory ratio of antibodies in serum samples, urine samples, and/or mucosal secretions. The dosages can be determined from animal studies, including animals that are not natural hosts to the bacterial species in question. For example, the animals can be dosed with a vaccine candidate, *e.g.*, a vaccine according to the invention, to partially characterize the immune response induced and/or to determine if any neutralizing antibodies have been produced. In addition, routine human clinical studies can be performed to determine the effective dose for humans.

[00172] Effective doses may be extrapolated from dose-response curves derived from *in vitro* and/or *in vivo* animal models. Ty21a is given every other day for three or four doses (depending on country of use), and the dose may be between about $2x10^9$ and $>10^{10}$ colony forming units (CFU) per dose. In one embodiment, dose spacing of every one to two months works better in some immunization schedules (*e.g.* in infants), and in another embodiment, doses as high as 10^{11} CFU may work better in developing countries (*i.e.*, trigger better protection that persists longer).

[00173] An immunologically effective amount, based upon human studies, would, in one embodiment, be sufficient to stimulate an acceptable level of protective immunity in a population. For some vaccines (in certain embodiments), this immunologically effective level would provide an 80% efficacy against a specific diarrheal disease. For other vaccines (in other embodiments), an immunologically effective amount would be one that protects against severe disease but may not protect against all symptoms of a disease.

[00174] In one embodiment, a vaccine (or other composition) according to the invention may also be administered to a subject at risk of developing a disorder, in an amount effective to prevent the disorder in the subject. As used herein, the phrase "effective to prevent the disorder" includes effective to hinder or prevent the development or manifestation of clinical impairment or symptoms resulting from the disorder, or to reduce in intensity, severity, and/or frequency, and/or delay of onset of one or more symptoms of the disorder.

Kits

[00175] Kits comprising the attenuated bacteria and acid resistance (*gad*) genes or a vaccine or a composition according to the invention are provided in an additional embodiment. Kits can include one or more other elements including, but not limited to,

instructions for use; other reagents, e.g., a diluent, devices or other materials for preparing the vaccine or composition for administration; pharmaceutically acceptable carriers; and devices or other materials for administration to a subject. Instructions for use can include instructions for therapeutic application (*e.g.*, DNA vaccination and protein boosting) including suggested dosages and/or modes of administration, *e.g.*, in a human subject, as described herein.

[00176] In another embodiment, a kit according to the invention can further contain at least one additional reagent, such as a diagnostic or therapeutic agent, *e.g.*, a diagnostic agent to monitor an immune response to the compositions or vaccines according to the invention in the subject, or an additional therapeutic agent as described herein (see, *e.g.*, the section herein describing combination therapies).

[00177] The existence of an immune response to the first dose of the immunoprotective composition may be determined by known methods (e.g. by obtaining serum from the individual before and after the initial immunization, and demonstrating a change in the individual's immune status, for example an immunoprecipitation assay, or an ELISA, or a bactericidal assay, or a Western blot, or flow cytometric assay, or the like) prior to administering a subsequent dose. The existence of an immune response to the first dose may also be assumed by waiting for a period of time after the first immunization that, based on previous experience, is a sufficient time for an immune response and/or priming to have taken place--*e.g.* 1, 2, 4, 6, 10 or 14 weeks. Boosting dosages of the immunoprotective composition will contain from about $5x10^6$ to $5x10^{11}$ organisms per patient, depending on the nature of the immunogen.

[00178] In one embodiment, the Ty21a(Gad) vaccine is used in humans at doses ranging from about 10^6 to about 5×10^{10} CFU and anywhere from about 2 to about 5 oral doses, spaced about every other day, as required for full immunity. Alternatively, vaccine dose spacing could be weekly or monthly, if more convenient.

[00179] The toxicity and therapeutic efficacy of the attenuated vaccines provided by the invention are determined using standard pharmaceutical procedures in cell cultures or experimental animals. One can determine the ED50 (the dose therapeutically effective in 50% of the population) using procedures presented herein and those otherwise known to those of skill in the art.

[00180] Various embodiments of the disclosure could also include permutations of the various elements recited in the claims as if each dependent claim was a multiple dependent claim incorporating the limitations of each of the preceding dependent claims as well as the independent claims. Such permutations are expressly within the scope of this disclosure.

[00181] While the invention has been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein without departing from the spirit and scope of the invention and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims. All references cited herein are incorporated in their entirety by reference.

EXAMPLES

[00182] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

[00183] RpoS is a transcription factor involved in AR1 that facilitates survival and aids in colonization and pathogenicity. It is triggered by acidification during the stationary phase. RpoS is responsible for managing stress sensitivity, including low pH, high osmolarity, oxidation, and heat. Acid environments can also damage the DNA through depurination and methylation, so DNA repair enzymes are necessary under these acidic conditions. However, the system described herein is independent of RpoS; therefore, the Gad system of *Shigella*, when functioning rpoS-independently, requires a stronger environmental signal such as anaerobiosis and growth on glucose.

[00184] Described herein are efforts to improve the current Ty21a vaccine by developing dissolvable compressed wafers/tablets/dissolvable membranes. These wafers/tablets/dissolvable membranes would be available to all ages, including infants, which is important due to the large capsule size of the current vaccine. The 3 Shigella GDAR genes *gadA*, *B* and *C* were synthesized and cloned into a low copy plasmid pNW129 under the control of an arabinose-inducible promoter to construct pGad. A series of acid resistance assays was performed to optimize the best conditions. Optimal conditions would be to survive at pH 2.5 for 2 hours in order to mimic the pH and gastric emptying time of a normal fasting stomach. Gastric acid of the stomach serves as a natural antibiotic barrier.

[00185] Thus, cells were pH-adapted at pH 5.5 in order to activate the Gad system and grown to stationary phase, to an OD between 2.8 and 3. During the low pH adaptation, the cells adapt to the acidic environment, synthesizing fatty acids. LB media was used instead of TSB to evaluate the effects of glucose. Glucose competes with the arabinose added as an inducing compound. The assay was performed under both aerobic and anaerobic conditions based on previous research.

Example 1. Expressing Gad proteins in Ty21a

[00186] *Construction of acid-resistant strains*

[00187] Standard molecular biology techniques were used for cloning throughout the experiments described herein. The restriction endonucleases and ligase were purchased from New England Biolabs (NEB) or Fermentas, and phusion polymerase (Fisher) was used for all PCRs. Plasmids pGad and pMD-SD were synthesized by GenTech, CA. In order to construct pMD-SV, the vexA gene (~ 1000bp) was PCR-amplified with primers prMND205.VexA.F (SEQ ID NO:11) and prMND206.VexA.R (SEQ ID NO:12) (Table 2) from Ty21a genomic DNA, digested with BamHI and NdeI, and ligated to pMD-SD that was digested with the same restriction enzymes and gel-extracted (to eliminate the tviD gene of pMD-SD).

[00188] In order to construct pMD-SD-Gad, the gad genes with arabinose promoter (~6500bp) were PCR amplified with primers prMND214.Gad.F.XhoI (SEQ ID NO:13) and prMND215.Gad.R.KpnI (SEQ ID NO:14) from plasmid pGad, digested with XhoI and KpnI, and ligated to pMD-SD that was digested with the same restriction enzymes. Ty21a vexA merodiploid (strain MD290) and Ty21a-Gad (strain MD 297) were constructed as described in Fig. 5. Ty21a-Gad was analyzed by PCR to confirm chromosomal integration, utilizing primers prMD92 and prMD124, and the resulting PCR product was sequenced.

[00189] *Bacterial strains, plasmids, and growth conditions*

[00190] The bacterial strains and plasmids utilized herein are described in Table 1. All of the strains were grown in Difco tryptic soy broth (TSB) or tryptic soy agar (TSA). Culture conditions used in this study are: TSB at pH 4.5 or 5.5 buffered with 2-(4-morpholino) ethane sulfonic acid (MES, Fisher), TSB at pH 6.5 or 7.5 buffered with 3-(4-Morpholino) propane sulfonic acid (MOPS, Fisher), or commercial TSB (pH 7.2). All TSB cultures contain 0.25% glucose unless mentioned otherwise. Cultures were supplemented with 0.01% galactose, 0.75% arabinose, and 1 mM glutamate, unless specified otherwise. All cultures were grown aerobically in a shaking incubator at 250 rpm and at 37°C.

[00191] For stationary phase cultures, a single colony from a fresh TSA plate was inoculated into 10 ml TSB and grown for either 18 or 24 hours. To obtain log phase cultures, overnight cultures were diluted 1:100 and grown until OD_{600} = 0.4 – 0.5. Plasmid-containing strains were selected in appropriate growth medium containing ampicillin (Amp; 100 μg/ml), chloramphenicol (Cam; 35μg/ml), or kanamycin (Kan; 30 μg/ml). All constructed plasmids and chromosomal integrants (*i.e.*, PCR products originating from the site of integration) were

sequenced, and the sequences were assembled and analyzed using Vector NTI suite 9.0 software (Invitrogen). Sequences are available under GENBANK accession numbers KJ870102 (SEQ ID NO:2) for the *tviD-vexA* insert region of Ty21a-Gad, KJ870100 (SEQ ID NO:1) for pGad, KJ870099 (SEQ ID NO:6) for pMD.SD, KJ870098 (SEQ ID NO:7) for pMD.SV and KJ870101 (SEQ ID NO:8) for pMD.SD.Gad.

[00192] Pre-growth conditions that may affect acid survival include the growth phase, the pH of the media, and the glucose (concentration) in the media.

[00193] Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Strains		
E. coli DH5α λpir	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Susan Gottesman lab
E. coli S17-1 λpir	TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir	Ronald Taylor lab
S. enterica serovar Typhi Ty2	Wt *rpoS*	Kopecko lab
S. enterica serovar Typhi Ty21a	*galE ilvD viaB (Vi-) H_2S- rpoS*	(Germanier & Furer, 1975)
Ty21a *vexA* merodiploid (MD290)	Two copies of *vexA* and FRT site	This study
Ty21a-Gad (MD297)	*S. flexneri gad* genes integrated into *tviD-vexA* on the chromosome, Kan^s, Amp^s, Cam^s	This study
S. flexneri 2457T CFS100	Avirulent isolate without the invasion plasmid	Shelly Payne lab
Plasmids		
pCP20	yeast Flp recombinase gene FLP, Cm^r, Amp^r, ts-rep[a]	(Cherepanov & Wackernagel, 1995)
pMD-SV	R6K, RP4, *vexA*	This study
pMD-SD	R6K, RP4, *tviD*	This study
pMD-SD-gad	R6K, RP4, *tviD, gadABC*	This study
pNW129	P15A rep, Kan^r	(Bonocora *et al.*, 2008)
pGad	gadABC cloned into pNW129	This study
pDB8	Tet resistant plasmid	Michael Levine lab

[a] ts-rep; temperature sensitive replication

[00194] Table 2. PCR Primers

Name	Sequence
prMND205.VexA.F	GATCA**CATATG**AAAAAAATCATCATATTACTAACGACATTTTTCC (SEQ ID NO:11)
prMND206.VexA.R	GATCA**GGATCC**TTAGAAAGAATTAGTGCCGCGGG (SEQ ID NO:12)
prMND214.Gad.F.XhoI	GATCA**CTCGAC**GCCGGAGGATCTGCCGTC (SEQ ID NO:13)
prMND215.Gad.R.kpnI	GATCA**GGTACC**CCAAGCTTGCATGCCTGCAG (SEQ ID NO:14)
prMD92	GTTGCGGTAATGGTATAACGAAATAACAGATAC (SEQ ID NO:15)
prMD124	CACGCAATATTTCAATGATGGCAAC (SEQ ID NO:16)

[00195] *Immunoblotting*

[00196] Bacterial strains were grown as described above, normalized based upon culture OD, and the appropriate volume centrifuged. Pelleted bacteria were re-suspended in lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM MgCl2, 10 mM CaCl2 and 1% SDS), sonicated for 10 sec using a Microson ultrasonic cell disruptor, centrifuged, and the supernatant was mixed with LDS sample buffer. Samples were separated by Bis-Tris PAGE. Standard Western blotting procedures were carried out with anti-Gad A/B antibody (Bhagwat, *et al.* 2004 *FEMS microbial letters* 234:139-147) for specific identification of Gad A/B proteins.

[00197] The Gad system of *Shigella* and *E. coli* is intricately regulated. The genes *gadA* (S4173) and *gadBC* (S1867 and S1868) are found about 2000 kb apart on the *S. flexneri* 2a strain 2457T (Wei, *et al.* 2003 *Infec and Immun* 71:2775-2786). These *gad* genes were cloned in tandem with their cognate ribosome binding sites under the control of an arabinose-inducible promoter (P_{araBAD}) in low copy plasmid pNW129 (Bonocora, *et al.* 2008 *Molec microbiol* 69:331-343) to create pGad (Fig. 1A). The intent was to make Gad proteins in ready response to added exogenous arabinose and, thus, free from the typical complex Gad-regulatory network. Since glucose is known to repress P_{araBAD} (Miyada, *et al.* 1984 *PNAS USA* 81:4120-4124), initial studies used LB media, which does not contain glucose. However, for later studies, TSB media was used, which contains 0.25% glucose, since better acid survival was seen with TSB than LB (data not shown). The TSB media was supplemented with 0.75% arabinose to counter any repression exerted by glucose. The Gad proteins were detected as early as 2 hours after addition of arabinose to the Ty21a pGad culture, and protein expression increased over time (Fig. 1B), indicating that cloned Gad expression is not sensitive to 0.25% glucose in TSB media in the presence of 0.75% arabinose.

Example 2. Extreme acid survival of Ty21a depends on growth phase.

[00198] *Acid resistance assay*

[00199] Five hundred microliters of TSB-pre-grown bacterial cultures were directly diluted into 10 ml of TSB-MES pH 2.5. Following incubation at 37°C with aeration, samples were retrieved at time 0, 1, 2 and 3 hours, and appropriate dilutions were immediately plated onto TSA plates to determine viable count. The % survival (input to output ratio) are presented as mean ± SEM of three or more independent experiments. Statistical analyses were performed by means of an unpaired t test using GraphPad Prism version 5. A P value of <0.05 (two tailed) was considered to be statistically significant (**).

[00200] To determine whether pGad can enhance the acid survival of Ty21a, selected bacteria were grown in regular TSB or TSB-MES pH 5.5 until OD600 0.4-0.5 (log phase, Fig. 2 A and B) or for 18 hours (stationary phase, Fig. 2C and 2D). When Ty21a pGad was pre-grown to log phase in regular TSB, cells did not survive the subsequent pH 2.5 acid challenge even for 1 hr (Fig. 2A). Of note, even log-phase *S. flexneri* lost 4 logs of viability over 3 hrs. However, when Ty21a pGad was pre-grown in TSB-MES pH 5.5, viability at pH 2.5 for 1 hr was slightly improved, but succumbed to pH 2.5 after 2 hours (Fig. 2B). Ty21a pNW129 and Ty2 did not show any substantive survival at pH 2.5 under either log phase pre-growth conditions. In contrast, *S. flexneri* showed low level survival (~ 0.01%) over 3 hrs at pH 2.5 following these growth conditions. Overall, bacteria in log phase showed poor acid survivability.

[00201] When selected bacteria were grown to stationary phase in regular or mildly acidic TSB prior to pH 2.5 acid challenge, the acid survival of Ty21a pGad was enhanced significantly (~ 5 logs) compared to Ty21a pNW129. Although, Ty21a pNW129 in stationary phase was more acid-resistant than that of log phase cells, most stationary phaseTy21a pNW129 also succumbed after 2 hours at pH 2.5 (Fig. 2C and 2D). The significant enhancement of acid resistance seen in Ty21a pGad is dependent, as expected, on the presence of arabinose (Fig. 2C). In the absence of arabinose, Ty21 pGad acid survival was comparable to that of Ty21a pNW129. These data demonstrate that the gad A, B, and C genes cloned under the control of an arabinose-inducible promoter in the low copy plasmid pNW129 are responsible for the significant enhancement of acid survival of Ty21 pGad.

[00202] Although a substantial amount of Gad proteins are expressed in log phase Ty21a pGad (4 hrs, Fig. 1B), this log phase-strain fails to withstand acid challenge (Fig. 2A, 2B). In striking contrast, Gad proteins provide significant protection to stationary phase Ty21a against acid challenge (Fig. 2C, 2D). These observations indicate that, in addition to Gad proteins, other ATR factors are expressed during stationary phase and under mildly

acidic pH that contribute to optimal acid resistance. Surprisingly, Ty2 pre-grown in regular TBS at neutral pH for 18 hours did not show any substantive survival under these conditions, whereas Ty21a pNW129 survived the acid challenge slightly better than Ty2 (Fig. 2C). Most importantly, wild-type Ty2 pre-grown at mildly acidic pH 5.5 showed enhanced acid survival (~3% after 3 hrs at pH 2.5; Fig. 2D), most likely due to induced ATRs. The acid survival of *S. flexneri* pre-grown to stationary phase was significantly enhanced relative to log phase, when pre-grown either at pH 5.5 or 7.2; however, pre-growth under mildly acidic conditions stimulated maximal subsequent survival at pH 2.5. Of note, in *Shigella*, the native Gad system is not induced until late exponential phase (Waterman, *et al.* 2003 *FEMS microbial letters* 225:155-160), and full ATR are apparently expressed under subneutral pH growth conditions.

Example 3. Effect of pre-growth media at pH ranging from 4.5 to 7.5 on extreme acid survival

[00203] A range of pH of the pre-growth media was next studied for effect on extreme acid survival, possibly through induction of stationary phase ATRs. Bacteria were pre-grown in TSB-MES pH 4.5, TSB-MES pH 5.5, TSB-MOPS pH 6.5, or regular TSB (unbuffered, pH 7.2) or TSB-MOPS pH 7.5 for 18 hours prior to the 3 hr. pH 2.5 acid challenge (Fig. 3). Ty21a pGad demonstrated good survival when bacteria were pre-grown in mildly acidic pH media and regular unbuffered media. Although Gad proteins are expressed at pH 7.5 (data not shown), the acid survival of Ty21a pGad decreased by ~3 logs at this growth pH. These data indicate that the ATR systems of Ty21a are not induced at pH 7.5; and that the induction of ATR, in addition to the Gad system, is important for acid survival. The negative control Ty21a pNW129, on the other hand, did not show substantive survival under any selected pH growth condition.

[00204] Although Ty2 showed good acid survival when pre-grown at mildly acidic pH, it showed very poor survival when pre-grown at pH 7.5 or 7.2 (regular TSB), indicating that the ATR of Ty2 is activated only at mildly acidic pH, but not at neutral or slightly basic pH. These data further emphasize the fact that Ty21a acid survival mechanisms, such as inducible ATRs, cannot be optimally induced (or ATR mechanisms that are intact are insufficient) to withstand extremely acidic conditions like the parent strain Ty2, due to mutations introduced by random mutagenesis during Ty21a construction. However, Ty21a pGad can achieve a remarkable level of acid resistance that is superior to Ty2 and comparable to Sf, but it requires both the expression of Gad proteins and the induction of ATR mechanisms under optimal growth conditions. Sf exhibited substantial survival under all pre-growth pH

conditions, including pre-growth in TSB-MOS pH 7.5. However, the *Shigella* systems necessary for optimal extreme acid survival were activated during pre-growth at pH 5.5 to stationary phase.

Example 4. Effect of pH changes in unbuffered TSB cultures

[00205] It is has been noted that Ty21a is more acid-sensitive than the parent strain Ty2, as demonstrated herein by comparing Ty21a pNW129 vs. Ty2 survival (Fig. 3). In a previous study comparing the acid survival of Ty21a to Ty2, the bacteria were pre-grown in mildly acidic TSB under anaerobic conditions for 18 hrs (Hone, *et al.* 1994 *Vaccine* 12:895-898). Surprisingly, when selected bacteria were pre-grown in regular unbuffered TSB prior to acid challenge, Ty21a pNW129 survival was ~3 logs superior to that of Ty2 after 1 hour (Fig. 2C and 2D). Thus, the ATRs of Ty21a, but not Ty2, appear to be induced in regular TSB. When this anomaly was investigated further, it was observed that the pH of the Ty21a pGad and Ty21a pNW129 cultures actually dropped below pH 6.5 after 18 hrs of growth, whereas the pH of the Ty2 culture remained close to the initial pH 7.2. Thus, this observed pH decrease for Ty21a strains grown in regular TSB likely activates remaining ATRs, whereas similarly grown Ty2 do not activate ATRs, because the pH remains at 7.2. However, pre-growth at pH 5.5 does induce ATRs of Ty2, which is more acid-resistant than similarly grown Ty21a pNW129 (Fig. 2D). Taken together, these observations demonstrate that the ATR of both Ty2 and Ty21a are induced by growth at mildly acidic pH. However, under optimal ATR-inducing conditions, Ty2 is considerably more acid-resistant than Ty21a (Fig. 2D).

Example 5. Glucose is important for induction of ATR.

[00206] Under anaerobic conditions, glucose in the media is fermented by *S.* Typhi, and the culture pH drops to ~5.5; however, under aerobic conditions, glucose is not fermented (Brenneman, *et al.* 2013 *J bacteriol* 195:3062-3072). Therefore, it was investigated whether glucose is responsible for the pH drop that was seen in Ty21a cultured in regular unbuffered TSB that contains 0.25% glucose. Selected bacteria were grown either in regular unbuffered TSB with glucose or TSB that does not contain glucose for 18 hours, and the pH of the cultures was determined (Fig. 4 A). In the absence of glucose in TSB, the pH drop was not observed with the cultured Ty21a strains, indicating that glucose metabolism is responsible for the pH drop. A recent study has reported that *galE* mutants (such as Ty21a) have global defects in carbohydrate metabolism (Lee, *et al.* 2009 *PNAS USA* 106:19515-19520), which may explain the pH differences observed during growth in standard TSB between Ty2 and Ty21a. For the *Shigella flexneri* culture in standard TSB, the

pH decreased at 18 hrs to < pH 6, and this pH drop was not dependent on glucose in the media.

[00207] In order to determine the overall influence of glucose on acid survival, selected cultures were grown in TSB with or without glucose prior to the pH 2.5 acid challenge. In standard TSB lacking glucose, Ty21a pGad survival at pH 2.5 was reduced by ~ 4 logs after 3 hours, and Ty21a pNW129 survival was reduced by 3 logs after 1 hour at pH 2.5 (Fig. 4B). These data indicate that glucose metabolism in the pre-growth media of Ty21a strains is important for increased acid survival by acidifying the media to activate the ATR and/or providing an energy source for the activation of ATR.

[00208] In order to decipher whether glucose metabolism simply acidifies the pre-growth media or plays a more direct role in activation of ATR, acid survival of selected bacteria was additionally compared in the presence and absence of glucose in TSB-MES pH 5.5 (Fig. 4C). Acid survival ability of Ty21a pGad and Ty2 was significantly reduced (4 to 5 logs after 3 hours) when glucose was absent in the media. Even Ty21a pNW129 acid survivability was reduced by > 4 logs after 1 hour at pH 2.5, when compared to glucose-grown Ty21a pNW129. These data clearly demonstrate that glucose in the pre-growth media is important both in direct activation of ATRs of Ty21a and Ty2, in addition to just acidifying the media of Ty21a cultures. In contrast, *S. flexneri* acid survival was largely unaffected by the presence of glucose in the pre-growth media. Nevertheless, *Shigella* acid-survival for 3 hrs was increased by ~1 log by pre-growth in glucose in standard TSB.

Example 6. Integration of *gad* genes into the Ty21a chromosome and removal of selectable antibiotic resistance

[00209] Since the Gad proteins must be stably expressed during vaccine manufacture, and pGad contains an antibiotic resistance marker that is objectionable to vaccine regulatory authorities, a technique was sought to insert the *gad* genes into the Ty21a chromosome, which would allow for subsequent removal of the antibiotic marker. A modified λ red recombination method has previously been reported, termed super-recombineering, to insert large (10-20 kb regions) *S. sonnei* O-antigen genes into the chromosome of Ty21a (Dharmasena, *et al.* 2013 *IJMM* 303:105-113). This technique was employed to insert *S. dysenteriae* 1, *S. flexneri 2a*, and *S. flexneri 3a* O-antigen genes into the Ty21a chromosome (unpublished data).

[00210] Described herein is a novel method to insert the *gad* genes into the Ty21a chromosome using a suicide vector and FRT (flippase recognition target) sites that allow for the subsequent removal of the antibiotic resistance cassette and any unwanted plasmid

regions with 100 % efficiency. Most importantly, this new method utilizes the host general recombination pathway (and does not require introduced λ Red enzymes). As schematically depicted in Fig. 5, two plasmid R6K-based suicide vectors were constructed, pMD-SV and pMD-SD, that require *trans* supply of the pir-encoded pi protein for replication. Although these plasmids can replicate in strains that express pi protein such as S17 λ*pir*, efficient plasmid suicide results upon transfer to Ty21a strains that lack *pir* (Rakowski, *et al.* 2013 *Plasmid* 69:231-242).

[00211] The donor strain S17λ*pir* with pMD-SV, which contains homology to the Vi operon *vexA* gene, was conjugated with Ty21a to transfer the pMD-SV plasmid into Ty21a. Kanamycin-resistant Ty21a with pMD-SV integrated into Vi operon were selected, and the region between the FRT sites was eliminated by expression of the flippase enzyme from plasmid pCP20 (Cherepanov, *et al.* 1995 *Gene* 158:9-14) to construct a *vexA* merodiploid strain MD290. Next, the *gad* genes, under control of an arabinose-inducible promoter, were cloned into pMD-SD (to generate pMD-SD-gad). Subsequent conjugal transfer of pMD-SD into MD290 and recombination at *tviD* resulted in integration of the entire pMD-SD-gad. Subsequent introduction of flippase resulted in removal of the plasmid replicon, *mob* sequences, KanR gene, and one *tviD* gene, but leaving the introduced *gad* genes. This Ty21a-Gad chromosomal insert strain MD297 contains the *gad* genes integrated between single copies of *tviD* and *vexA* of the Ty21a Vi operon. As noted above, these constructs were confirmed by PCR and DNA sequence analyses.

Example 7. Gad protein expression and acid survival of Ty21a-Gad

[00212] Next, Gad protein expression of the chromosomal integrant (Ty21a-Gad) was compared to that of the plasmid construct (Ty21a pGad). Bacteria were grown in TSB-MES pH 5.5 with or without glucose for 18 or 24 hours prior to acid challenge. The arabinose inducible promoter, P_{araBAD}, which is driving expression of the Gad proteins, is typically repressed by glucose. However, the Gad protein expression of the plasmid construct was not repressed by glucose at 18 hours or 24 hours and expressed high levels of Gad protein even in the presence of glucose. In contrast, the chromosomal integrant was sensitive to glucose in the media. Although Ty21a-Gad did not express Gad proteins at 18 hours in the presence of glucose, a moderate amount of Gad protein expression was seen at 24 hours.

[00213] Since *Salmonella* catabolize glucose as its main energy source (Dandekar, *et al.* 2012 *Frontiers in microbial* 3:164), it is plausible that the glucose in the media is gradually depleted, and Gad protein expression is increased over time. Since glucose is essential for the optimum ATR of Ty21a, growing the bacteria for 24 hours would allow Gad

protein expression in the presence of glucose. However, in the absence of glucose, a moderate level of Gad protein was expressed even at 18 hours. Overall, Gad protein expression from the chromosome was less, compared with that from the low copy plasmid with about 5 copies per cell.

[00214] The acid resistance afforded by the chromosomal integrant and plasmid construct in TSB-MES pH 5.5 grown for 18 hours and 24 hours was also compared. Consistent with level of Gad protein expression, Ty21a-Gad acid survival showed poor survival and was comparable to that of Ty21a at 18 hours, but the acid survival improved significantly (~3 logs) when the bacteria were grown for 24 hours prior acid challenge. This indicates that the acid survival of Ty21-Gad depends on level of Gad protein expression.

Example 8. *In vitro* intramacrophage survival

[00215] *Intramacrophage survival assay*

[00216] The U937 cell line was obtained from ATCC. The intramacrophage survival assay for *S.* Typhi was performed as previously described (Dragunsky, *et al.*, 1989 *J biol standardization* 17:353-360) with slight modifications. The cells were grown in RPMI 1640 (Gibco, NY) supplemented with 5 % fetal bovine serum (FBS; Gibco, NY) and 50 U/ml penicillin-streptomycin (Life technologies, NY) at 37°C with 5% CO_2. Cells were centrifuged at 58 X g for 10 min, resuspended in fresh 37°C-prewarmed RPMI 1640 medium + FBS without antibiotics, adjusted to a concentration of ~$1x10^6$ cells per ml, and 10 ml of cells were added to a 50 ml tube. After 24 hours, bacterial cultures were centrifuged, and the bacterial pellets were resuspended in RPMI 1640 + FBS. The bacteria were added to U937 cells at a ratio of ~100 bacteria per U937 cell (time 0).

[00217] After incubation at 37°C for 2 hours, the infected U937 cells were centrifuged as before, washed 2 x with phosphate buffered saline (PBS), and resuspended in RPMI 1640 medium + FBS containing 250 μg/ ml gentamicin in order to kill extracellular bacteria. After another 2 hours (time 4 hours), the cells were resuspended in 20 ml fresh RPMI medium containing 25 μg/ml gentamicin to prevent extracellular growth of any released bacteria. Additional PBS-washing of the cells was done at 24 hours post-infection. For viable bacterial count determinations at time 0, 4 and 24 hours, the infected macrophages were lysed with 0.1% Triton X-100 in PBS. After 3 min, cell lysates were serially diluted 10-fold in PBS, and aliquots were plated onto TSA plates to assess bacterial CFU. All assays were repeated at least three times on different days. The results are presented as the mean ± SEM of all replicates. Statistical analyses were performed by means of an unpaired t test using

GraphPad Prism version 5. A P value of <0.05 (two tailed) was considered to be statistically significant (**).

[00218] *S.* Typhi is a human-specific pathogen that does not naturally infect any animal species including monkeys. The ability to survive and replicate within macrophages/monocytes is thought to be one of the major pathogenesis determinants for *S.* Typhi, which helps them disseminate via the systemic circulation (Daigle 2008 *J infec develop countr* 2:431-437). *S.* Typhi survive and replicate within macrophages by adapting to the conditions within fused phagolysosomes, which include low pH, as they do not inhibit phagosome-lysosome fusion (Ishibashi, *et al.* 1995 *FEMS immunol and med microbiol* 12:55-61). Since there is no natural disease animal model for this organism, and a key attenuating feature of Ty21a involves its inability to survive in macrophages, cultured human macrophage cell lines can be used as a surrogate model to analyze relative attenuation.

[00219] Previously, when infectivity of Ty21a, in comparison with Ty2, was evaluated using the human monocyte-macrophage cell line U937, Ty21a showed markedly reduced entry and very low intramacrophage survival, whereas Ty2 entered macrophage at a higher rate and showed 100% survival and robust replication within U937 cells (Dragunsky, et al. 1989 J boil standardization 17:353-360). The infectivity of Ty21a, Ty21a pGad, and Ty21a-Gad was determined in comparison with Ty2, using the human monocyte-macrophage cell line U937. Ty21a, Ty21a pGad, and Ty21a-Gad showed very low entry (relative to Ty2) and could not replicate within macrophage. In contrast, Ty2 entered macrophage at a higher efficiency and showed robust replication within these macrophages over 24 hrs. These data indicate that addition of the Gad genes in strains Ty21a pGad orTy21a-Gad did not alter the known attenuation of Ty21a for macrophages.

[00220] Thus, the acid survival of Ty21a was greatly enhanced by expression of *Shigella* Gad AR proteins and bacterial growth under ATR inducing conditions. When Ty21a were grown to stationary phase in mildly acidic media containing glucose, ATR was optimally induced, but survival decreased 6 logs at pH 2 over 3 hrs. When Gad proteins were expressed from an arabinose-inducible promoter under optimal ATR conditions, Ty21 pGad acid survival was enhanced by 5 logs over a 3 hr period at pH 2.0, and survival equaled that seen with the highly acid-resistant *Shigella flexneri* control. The parent Ty2 strain was more acid-tolerant than Ty21a, probably due to mutations introduced during Ty21a construction. However, the acid survival of parent strain Ty2 was strictly dependent on ATR inducing conditions, and absence of any one of these optimal conditions resulted in a 4-5 log reduction in acid survival. These results suggest that the infectious dose of *S.* Typhi may vary greatly

depending on nutritional sources and environmental conditions. Although the infectious dose of *S.* Typhi is thought to range from ~105 to 109 cfu based upon volunteer studies, epidemiological data estimate a lower dose of 103 cfu from water-borne outbreaks (Blaser, *et al.* 1982 *Reviews infec dis* 4:1096-1106).

[00221] RpoS plays an important role in triggering general stress responses to protect bacteria against environmental challenges that include nutrient starvation, variations in temperature, osmolarity, or pH (Battesti, et al. 2011 *Ann rev microbiol* 65: 189-213). In *S. Typhimurium*, there are two distinct ATR systems in the stationary phase. One system is dependent on RpoS, and the other is RpoS-independent and acid-inducible. The ASPs induced during log phase ATR are regulated by RpoS, PhoP/Q and Fur. However, the stationary phase ATR provides more sustained and better protection than log phase ATR (Foster 1995 *Critical rev microbiol* 21: 215-237).

[00222] Results described herein demonstrate that Ty2 and Ty21a strains in stationary phase are more acid-resistant than that of log phase. Since Ty2 and Ty21a strains express an elongated, suboptimal RpoS, the major acid survival mechanism that is active under aerobic conditions is the acid-inducible, RpoS-independent ATR. Ty21a shows poor acid survival even under the herein-defined ATR-optimal growth conditions and may not be able to express all the ASPs that contribute to RpoS-independent ATR due to uncharacterized mutations. Nevertheless, acid survival of Ty21a is enhanced by an impressive 5 logs with the expression of Gad proteins and induction of remaining ATR mechanisms.

[00223] Since Ty21a pGad showed better acid survival under aerobic conditions that are more desirable for vaccine manufacture, acid resistance was studied more thoroughly under aerobic conditions. The acid survival of *Salmonella* mediated by the native arginine (AR3), lysine (AR4), or ornithine (AR4) decarboxylases are strictly dependent on anaerobic conditions, and these decarboxylases are not typically expressed under aerobic conditions (Brenneman, *et al.* 2013 *J bacteriol* 195: 3062-3072; Viala, *et al.* 2011 *PloS one* **6**: e22397).

[00224] The Gad system described in this study consists of paralogous GadA and/or GadB decarboxylases and inner membrane bound antiporter GadC. GadA and GadB can form 330 kDa hexamers assembled from trimerization of GadA(B) dimers. When the bacterial cells are exposed to extreme pH (~2.5), the cytoplasmic pH drops to ~ pH 4.5, and the N-terminal domain of these two isozymes undergoes a conformational change that allows interaction with the inner membrane to form the active form. The active enzymes convert glutamate to gamma-amino butyric acid (GABA) and carbon dioxide (CO2) in a reaction that consumes a cytoplasmic proton. The inner membrane antiporter GadC transports GABA out

of the cell in exchange for more glutamate. However, when the cytoplasmic pH increases back to neutral, GadAB is returned to an inactive form (Zhao, *et al.* 2010 *Biochem and cell biol* 88: 301-314).

[00225] The intent to express Gad proteins in Ty21a during vaccine manufacture is, therefore, described herein. During oral delivery of vaccine strain as a dried wafer, Gad decarboxylase (*i.e.*, GadA and/or GadB) expression will be maintained by the addition of inducible arabinose, and Gad activity will only be activated when the cells are exposed to low pH. Although Gad expression in Ty21a significantly improved extreme acid survival at pH 2.0, it did not provide any detectable advantage for intramacrophage survival. Since macrophage survival is one of the major virulence determinants of *S.* Typhi, it appears unlikely that the expression of Gad proteins alters Ty21a attenuation (or the safety of the vaccine). Acid-resistant Ty21a delivered in a rapidly dissolvable wafer format to the sublingual lymphoid tissue would be a very attractive oral platform system for delivery of diverse foreign antigens from numerous infectious agents. Further, this acid-resistance system may be extendable to other live oral vaccine strains (*e.g.* attenuated *Salmonella*, *Vibrio cholerae*) to improve oral delivery formats.

[00226] It is, thus, shown herein that pGad improves extreme acid (pH 2.5) survival of Ty21a by about 5 logs when ATR is induced. Furthermore, both pGad and ATR are necessary to achieve good acid survival that is comparable to *Shigella*. Finally, pre-growth conditions important for activation of ATR in Ty2 and Ty21a include: stationary phase, mildly acidic pH, and glucose.

Sequences

[00227] Sequences referred to herein include: SEQ ID NO:1 (GENBANK accession no. KJ870100; pGad), SEQ ID NO:2 (GENBANK accession No. KJ870102; gad genes inserted under control of arabinose into chromosome of Ty21a = "Ty21a-gad" (tviD-vexA insert region of Ty21a-Gad)), SEQ ID NO:3 (*gadA* from *Shigella flexneri* 2a strain 2457T), SEQ ID NO:4 (*gadB* from *Shigella flexneri* 2a strain 2457T), SEQ ID NO:5 (*gadC* from *Shigella flexneri* 2a strain 2457T), SEQ ID NO:6 (GENBANK accession no. KJ870099; pMD.SD), SEQ ID NO:7 (GENBANK accession no. KJ870098; pMD.SV), SEQ ID NO:8 (GENBANK accession no. KJ870101; pMD.SD.Gad), SEQ ID NO:9 (pMD.SD.lpp.gadABC (plasmid in which gadA DNA sequence is codon-optimized, for integration into Ty21a chromosome at Vi operon (in between tviD and vexA))), SEQ ID NO:10 (lpp.gad.integrated into tviD.vexA (gad genes under control of lpp promoter integrated into tviD-vexA region of Vi operon)), and SEQ ID NOs:11-16 (described in Table 2, above).

[00228] The description of the present invention has been presented for purposes of illustration and description, but is not intended to be exhaustive or limiting of the invention to the form disclosed. The scope of the present invention is limited only by the scope of the following claims. Many modifications and variations will be apparent to those of ordinary skill in the art. The embodiment described and shown in the figures was chosen and described in order to best explain the principles of the invention, the practical application, and to enable others of ordinary skill in the art to understand the invention for various embodiments with various modifications as are suited to the particular use contemplated.

CLAIMS

1. An immunoprotective composition comprising attenuated bacteria expressing i) at least one of glutamic acid decarboxylase (Gad) proteins GadA and GadB, and ii) antiporter GadC, or functional variants thereof,

wherein the attenuated bacteria comprise an expression cassette comprising two or three glutamic acid decarboxylase (Gad) system genes *GadA* and/or *GadB*, and *GadC*, or functional variants thereof, isolated from *Shigella* or certain *Escherichia coli* strains and operably linked to transcriptional promoter signals,

wherein the attenuated bacteria are rendered acid-resistant as a result of the expression of the Gad decarboxylase and antiporter proteins.

2. The immunoprotective composition of claim 1, wherein said attenuated bacteria are selected from the group consisting of *Campylobacter jejuni, Campylobacter coli, Listeria monocytogenes, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis. Escherichia coli, Shigella flexneri, Shigella sonnei, Shigella dysenteriae, Shigella boydii, Helicobacter pylori, Helicobacter felis, Gastrospirillum hominus, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Bacteroides fragilis, Clostridium difficile, Salmonella typhimurium, Salmonella typhi, Salmonella gallinarum, Salmonella pullorum, Salmonella choleraesuis, Salmonella enteritidis, Salmonella spp., Streptococcus gordonii, Lactobacillis sp., Klebsiella pneumoniae, Enterobacter cloacae, and Enterococcus faecalis.*

3. The immunoprotective composition of claim 2, wherein the attenuated bacteria are selected from the group consisting of *Salmonella typhi* Ty21a, *Vibrio cholerae* CVD103HgR, and *Vibrio cholerae* Peru15.

4. The immunoprotective composition of claim 1, wherein said attenuated bacteria are probiotic bacteria selected from the group consisting of actinobacteria, proteobacteria, firmicutes, and bacteroidetes.

5. The immunoprotective composition of claim 4, wherein the probiotic bacteria are selected from the group consisting of *Bifidobacterium*, *Lactobacillus spp.*, *Escherichia coli*, and *Lactococcus lactis*.

6. The immunoprotective composition of claim 1, wherein said cassette comprises SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5 or a functional variant thereof, each operably linked to a promoter or at least two or all three linked together and operably linked to a promoter.

7. The immunoprotective composition of claim 6, wherein the promoter is an arabinose-inducible promoter.

8. The immunoprotective composition of claim 6, wherein the promoter is a constitutive promoter.

9. The immunoprotective composition of claim 1, wherein the Gad proteins are expressed from a recombinant plasmid.

10. The immunoprotective composition of claim 9, wherein the recombinant plasmid contains at least one selectable marker.

11. The immunoprotective composition of claim 1, wherein the Gad proteins are expressed from a chromosomal integration site.

12. The immunoprotective composition of claim 1, further comprising a pharmaceutical diluent.

13. A plasmid comprising a polynucleotide encoding the genes i) *GadA* and/or *GadB,* and ii) *GadC*, or functional variants thereof, from a *Shigella* species operably linked to transcriptional promoter signals.

14. The plasmid of claim 13, wherein the plasmid encodes glutamic acid decarboxylase (Gad) proteins i) GadA and/or GadB, and ii) GadC, or functional variants thereof.

15. Acid-resistant attenuated bacteria, wherein the bacteria express: i) at least one of glutamic acid decarboxylase (Gad) protein GadA and GadB, and ii) antiporter GadC, or functional variants thereof, wherein the attenuated bacteria comprise an expression cassette

comprising two or three glutamic acid decarboxylase system (Gad) genes *GadA* and/or *GadB*, and *GadC*, or functional variants thereof, isolated from a *Shigella* species and operably linked to transcriptional promoter signals, wherein the expression of the three Gad proteins renders the attenuated bacteria acid-resistant.

16. The acid-resistant attenuated bacteria of claim 15, wherein the bacteria are induced by growth in Tryptic Soy Broth at about pH 5.5 in the presence of about 0.75% arabinose.

17. A method for preparing acid-resistant attenuated bacteria, comprising growing bacteria comprising an expression cassette comprising two or three glutamic acid decarboxylase (Gad) genes *GadA* and/or *GadB*, and *GadC*, or functional variants thereof, isolated from a *Shigella* species or *Escherichia coli* and operably linked to transcriptional promoter signals in a culture medium having subneutral pH under aerobic culture conditions.

18. The method of claim 17, wherein the subneutral pH lies between about pH 4.0 and about pH 6.8.

19. The method of claim 18, wherein the subneutral pH lies at about 5.5.

20. The method of claim 17, wherein the culture medium comprises glucose.

21. The method of claim 17, wherein the culture medium comprises arabinose.

22. The method of claim 17, wherein the culture medium comprises glutamate.

23. A vaccine comprising the immunoprotective composition of any one of claims 1-12 and a pharmaceutically acceptable carrier.

24. The vaccine of claim 23, wherein the vaccine is formulated as a compressed wafer.

25. The vaccine of claim 23, wherein the vaccine is formulated as a tablet.

26. The vaccine of claim 23, wherein the vaccine is formulated as a dissolvable membrane.

27. The vaccine of claim 23, wherein the vaccine is formulated as a chewable gum.

28. The vaccine of claim 24, wherein the compressed wafer comprises bicarbonate.

29. The vaccine of claim 28, wherein the bicarbonate is in an amount lower than the amount that would be required for full buffering of the stomach.

30. The vaccine of claim 24, wherein the wafer is formulated with arabinose and glutamate.

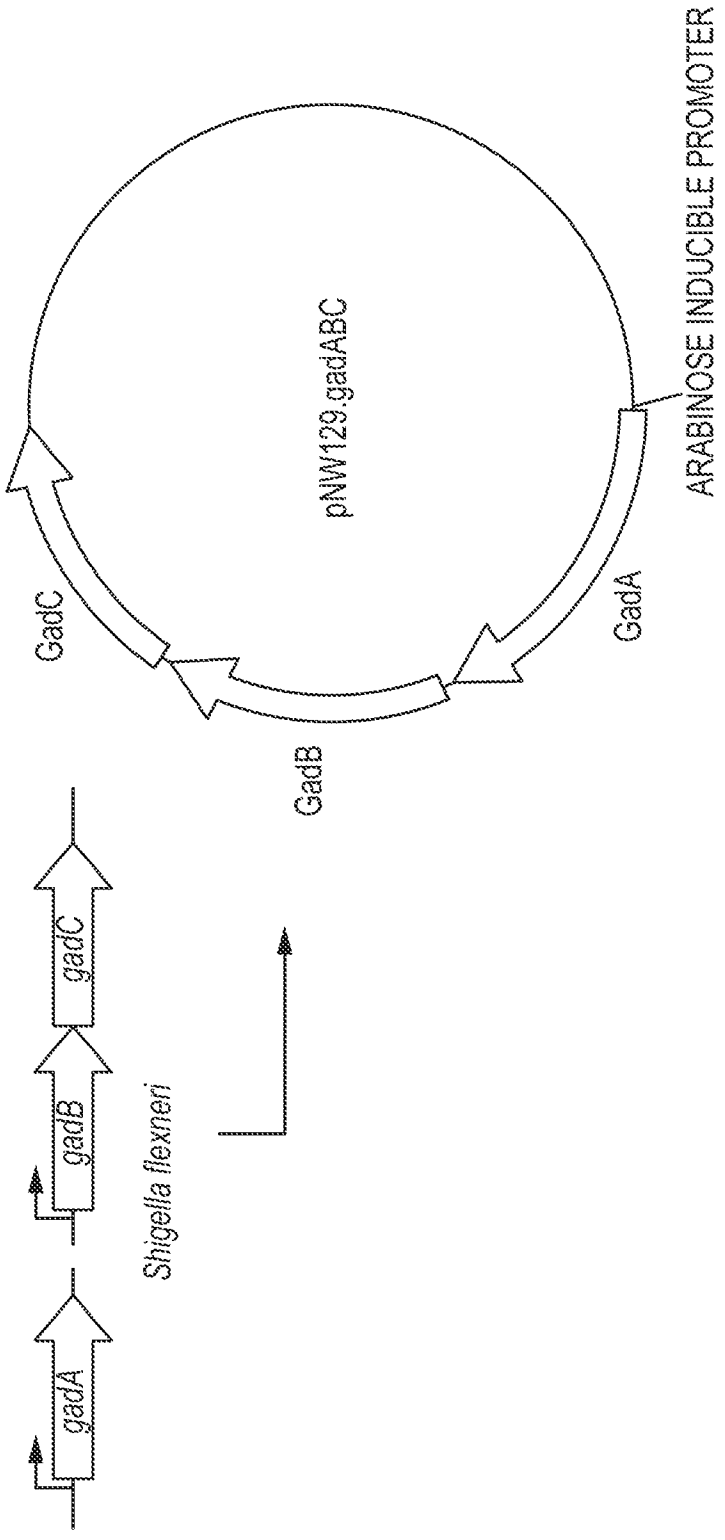

gadA
gadB
gadC
Shigella flexneri
GadB
GadC
pNW129.gadABC
GadA
ARABINOSE INDUCIBLE PROMOTER

FIG. 1A

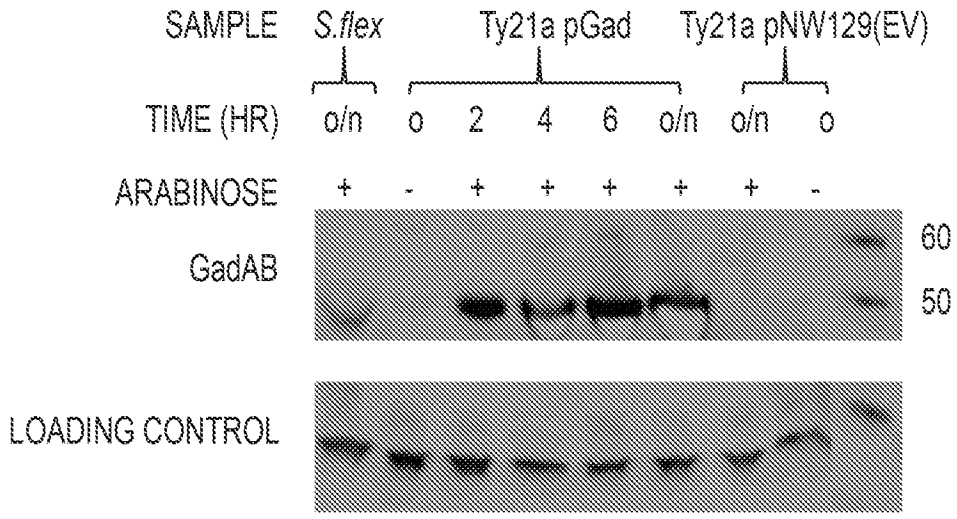

SAMPLE
S.flex
Ty21a pGad
Ty21a pNW129(EV)
TIME (HR)
o/n
o
2
4
6
o/n
o/n
o
ARABINOSE
+
-
+
+
+
+
+
-
GadAB
60
50
LOADING CONTROL

FIG.1B

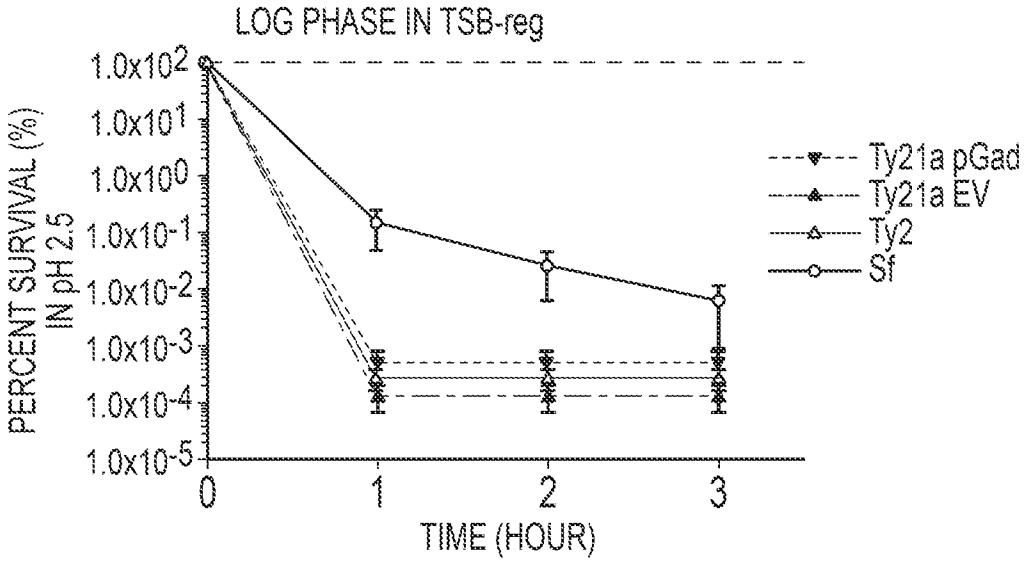

LOG PHASE IN TSB-reg
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
0
1
2
3
TIME (HOUR)
Ty21a pGad
Ty21a EV
Ty2
Sf

FIG.2A

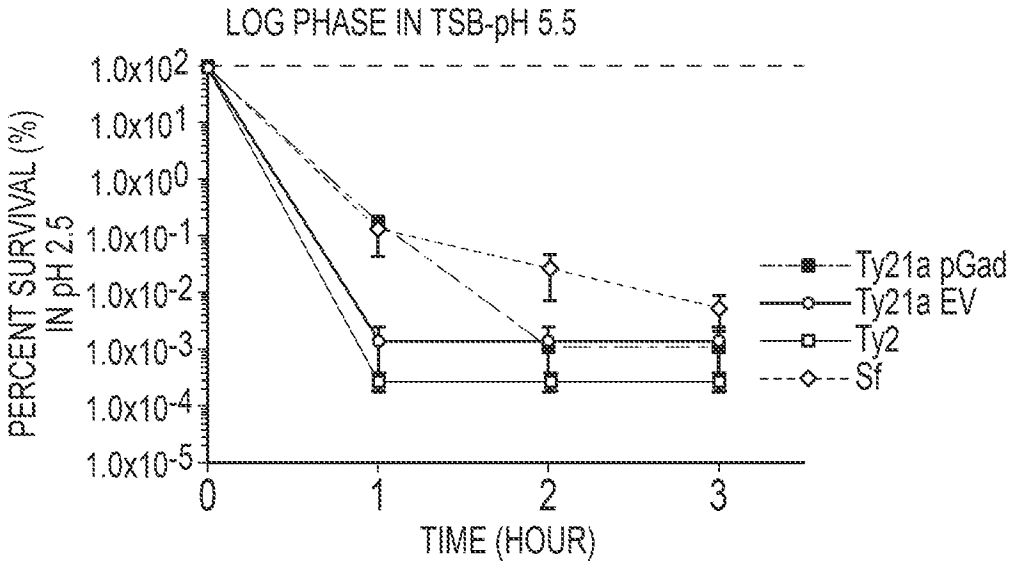

LOG PHASE IN TSB-pH 5.5
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
0
1
2
3
TIME (HOUR)
Ty21a pGad
Ty21a EV
Ty2
Sf

FIG.2B

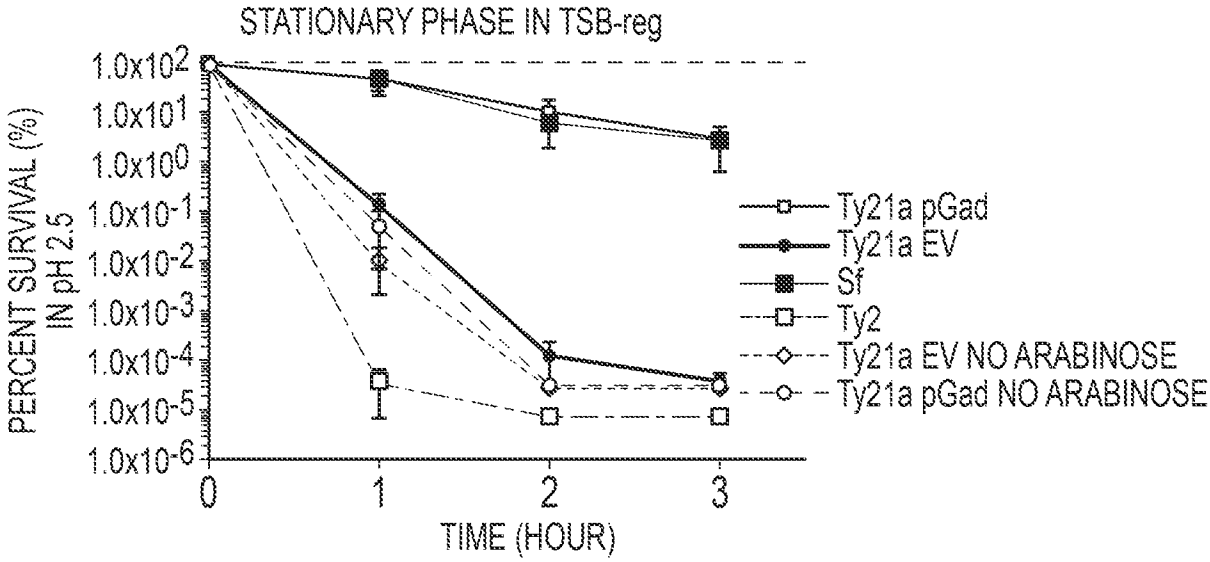

STATIONARY PHASE IN TSB-reg
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
0
1
2
3
TIME (HOUR)
Ty21a pGad
Ty21a EV
Sf
Ty2
Ty21a EV NO ARABINOSE
Ty21a pGad NO ARABINOSE

FIG.2C

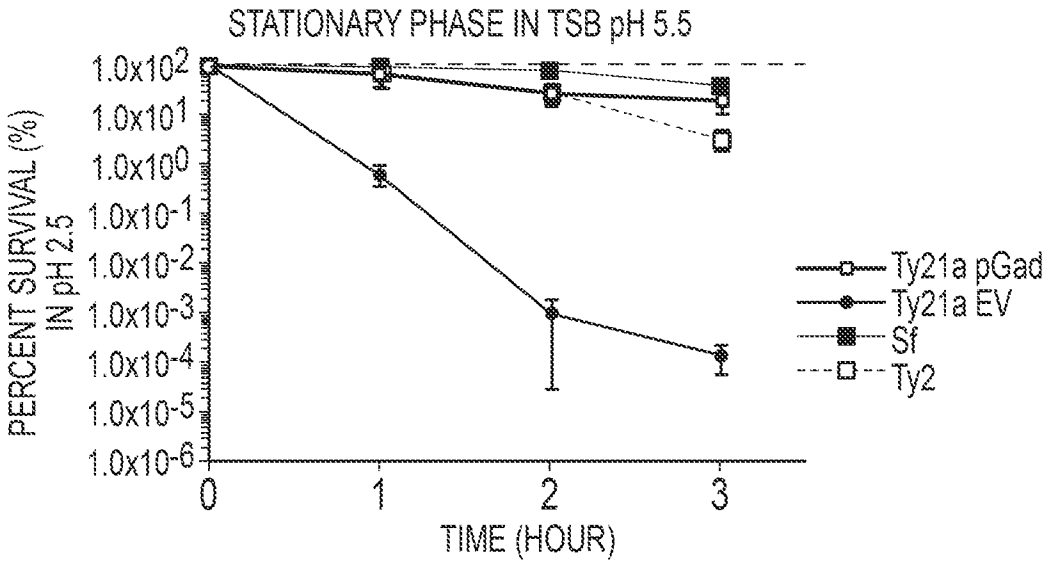

STATIONARY PHASE IN TSB pH 5.5
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
0
1
2
3
TIME (HOUR)
Ty21a pGad
Ty21a EV
Sf
Ty2

FIG.2D

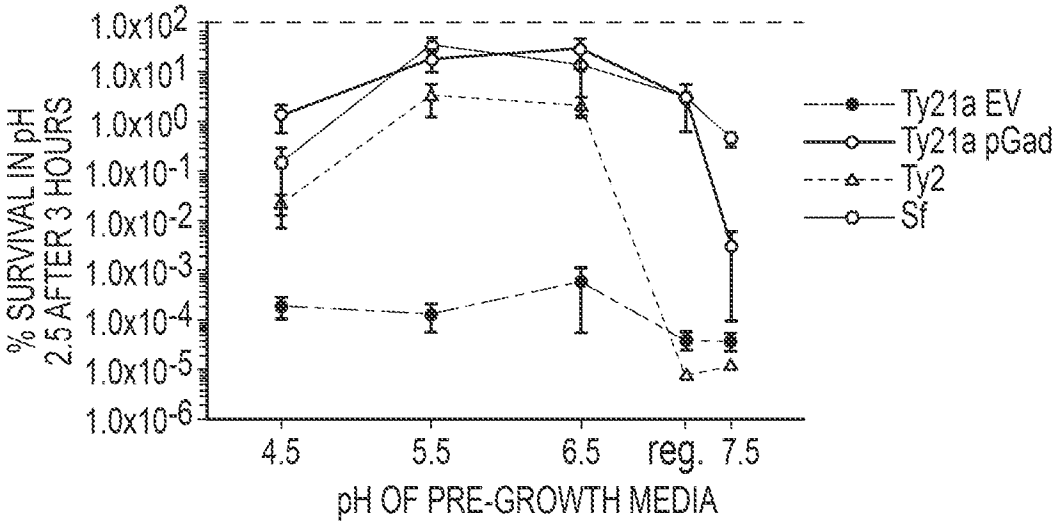

% SURVIVAL IN pH 2.5 AFTER 3 HOURS
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
4.5
5.5
6.5
reg. 7.5
pH OF PRE-GROWTH MEDIA
Ty21a EV
Ty21a pGad
Ty2
Sf

FIG.3

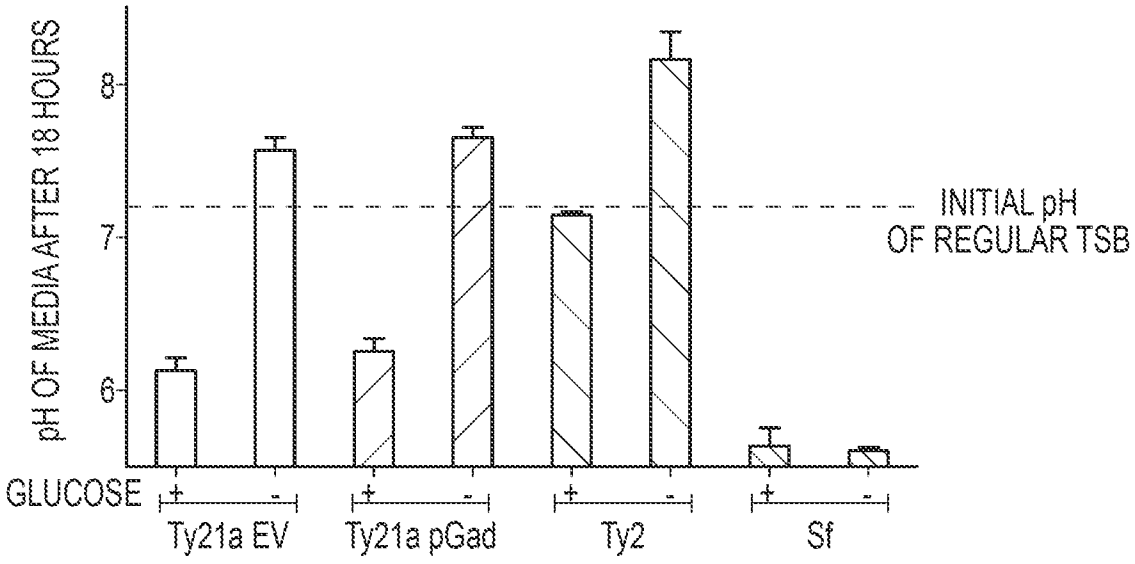

pH OF MEDIA AFTER 18 HOURS
8
7
6
INITIAL pH
OF REGULAR TSB
GLUCOSE
+
-
Ty21a EV
Ty21a pGad
Ty2
Sf

FIG.4A

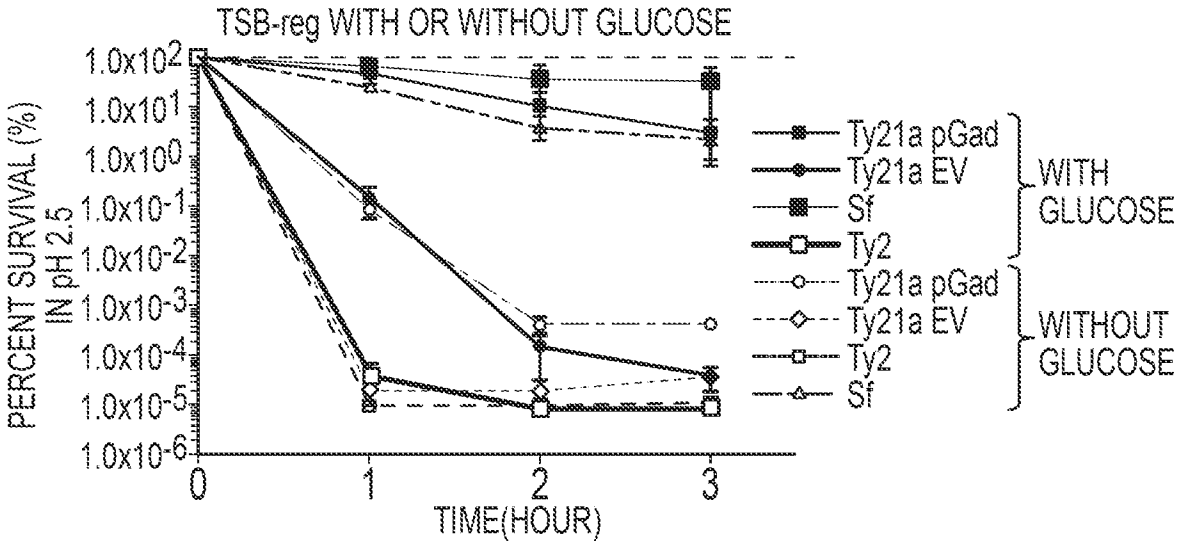

TSB-reg WITH OR WITHOUT GLUCOSE
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
0
1
2
3
TIME(HOUR)
Ty21a pGad
Ty21a EV
Sf
Ty2
WITH GLUCOSE
Ty21a pGad
Ty21a EV
Ty2
Sf
WITHOUT GLUCOSE

FIG.4B

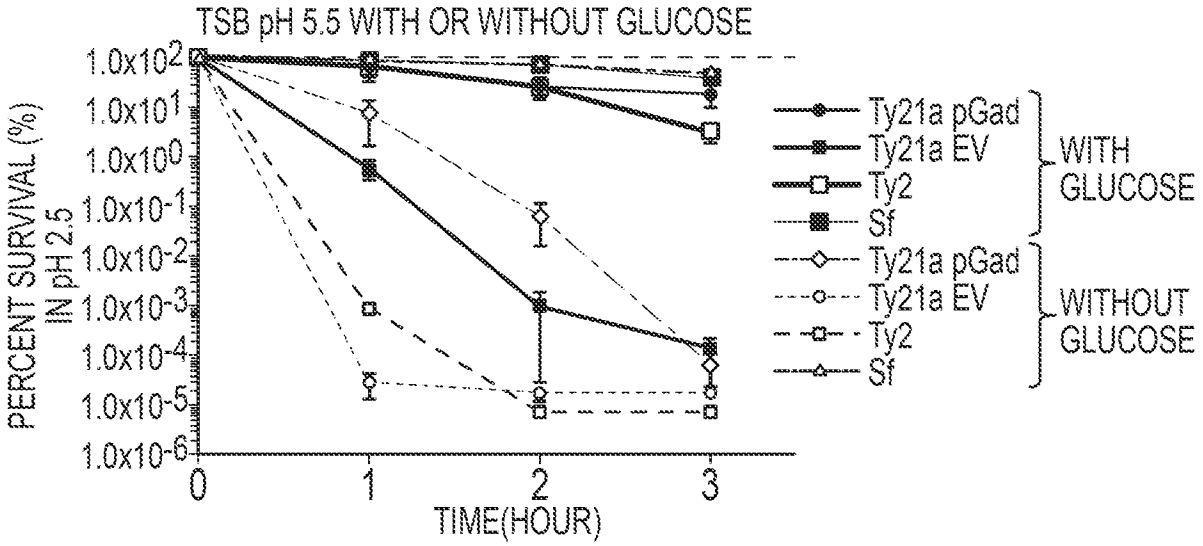

TSB pH 5.5 WITH OR WITHOUT GLUCOSE
PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
0
1
2
3
TIME(HOUR)
Ty21a pGad
Ty21a EV
Ty2
Sf
WITH GLUCOSE
Ty21a pGad
Ty21a EV
Ty2
Sf
WITHOUT GLUCOSE

FIG.4C

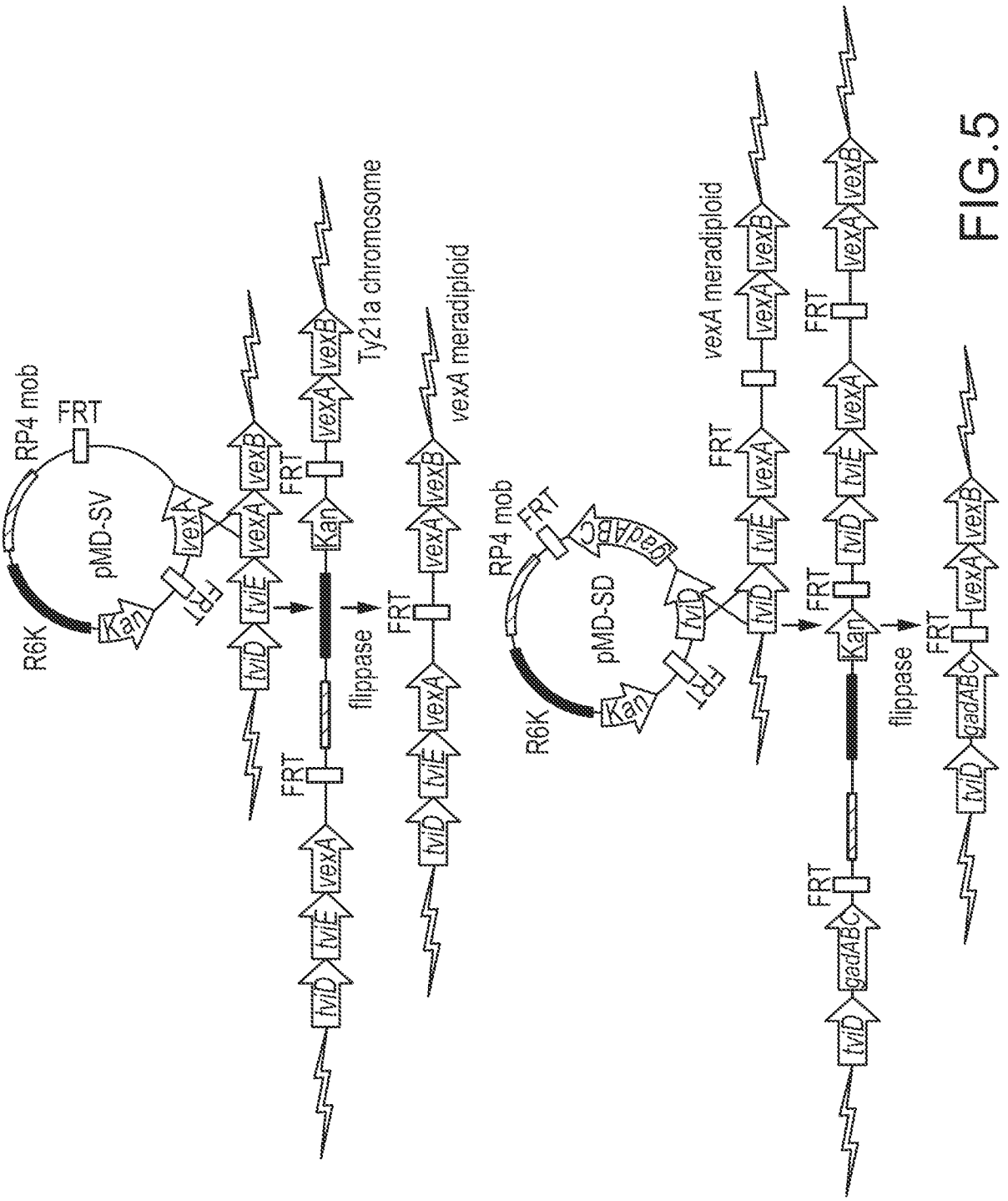

RP4 mob
FRT
pMD-SV
R6K
Kan
FRT
vexA
tviD
tviE
vexA
vexB
FRT
Kan
vexA
vexB
Ty21a chromosome
flippase
FRT
tviD
tviE
vexA
vexA
vexB
vexA meradiploid
RP4 mob
FRT
pMD-SD
R6K
Kan
FRT
gadABC
tviD
vexA meradiploid
FRT
tviD
tviE
vexA
vexB
FRT
Kan
tviD
tviE
vexA
vexA
vexB
FRT
flippase
FRT
tviD
gadABC
vexA
vexB

FIG.5

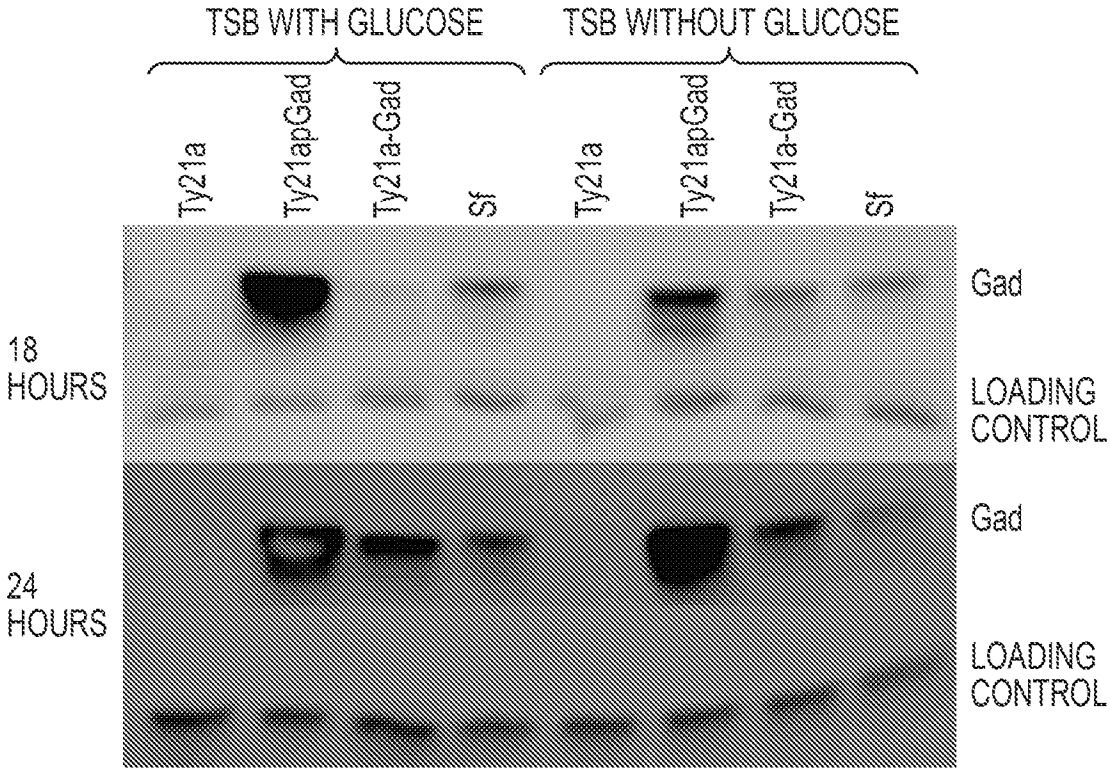

TSB WITH GLUCOSE
TSB WITHOUT GLUCOSE
Ty21a
Ty21apGad
Ty21a-Gad
Sf
Ty21a
Ty21apGad
Ty21a-Gad
Sf
18 HOURS
Gad
LOADING CONTROL
24 HOURS
Gad
LOADING CONTROL

FIG.6A

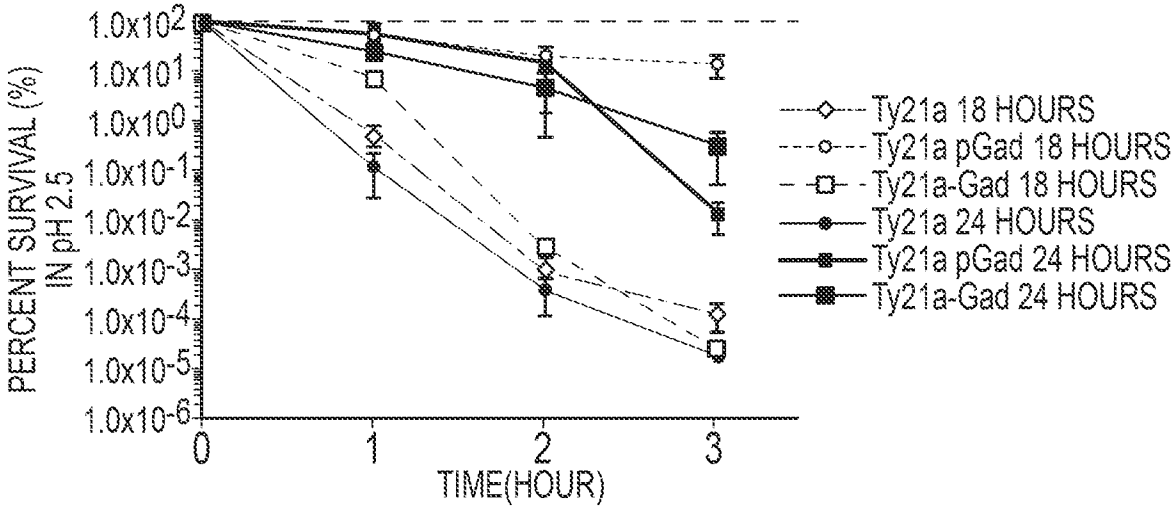

PERCENT SURVIVAL (%) IN pH 2.5
1.0x10^2
1.0x10^1
1.0x10^0
1.0x10^-1
1.0x10^-2
1.0x10^-3
1.0x10^-4
1.0x10^-5
1.0x10^-6
0
1
2
3
TIME(HOUR)
Ty21a 18 HOURS
Ty21a pGad 18 HOURS
Ty21a-Gad 18 HOURS
Ty21a 24 HOURS
Ty21a pGad 24 HOURS
Ty21a-Gad 24 HOURS

FIG.6B

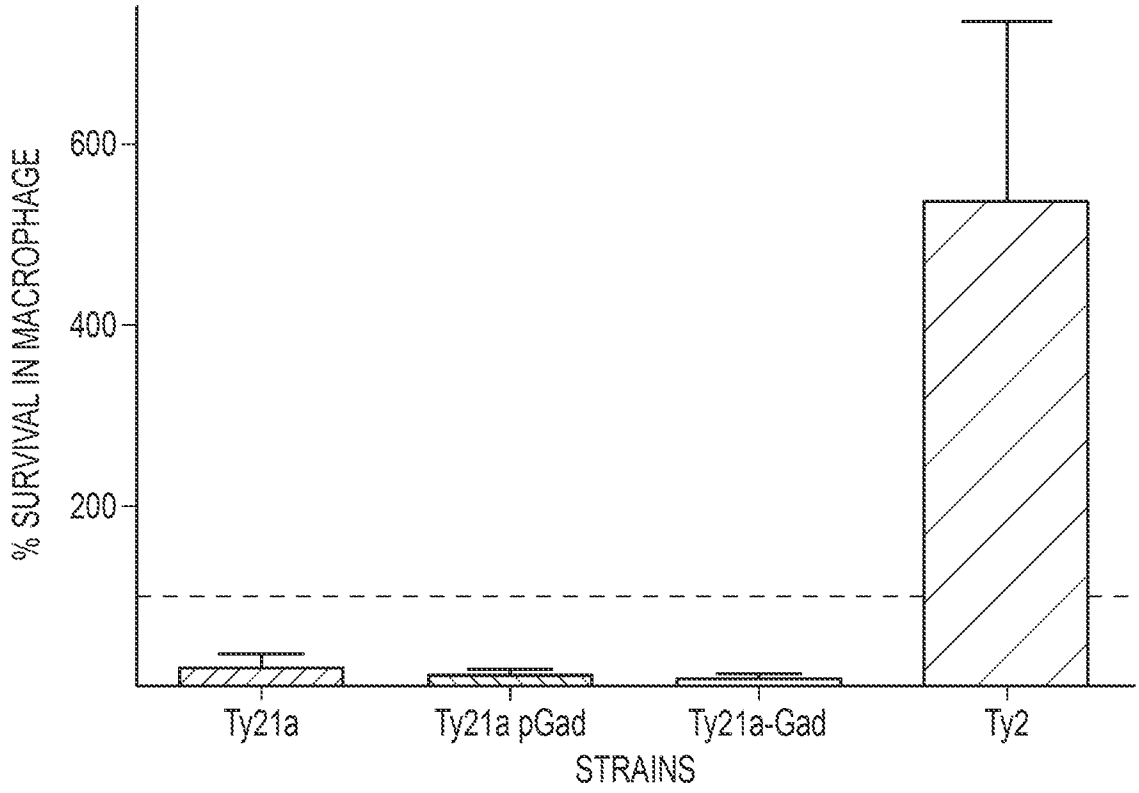

% SURVIVAL IN MACROPHAGE
600
400
200
Ty21a
Ty21a pGad
Ty21a-Gad
Ty2
STRAINS

FIG.7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/049933

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
- ☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
- ☐ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/049933

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/20(2006.01)i, A61K 39/02(2006.01)i, A61P 31/04(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 1/20; C12P 13/22; C07H 21/00; C12R 1/19; C07K 14/245; C12Q 1/68; C40B 30/04; A61K 39/02; A61P 31/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:immunoprotective composition, acid resistance, glutamic acid decarboxylase, GadA, GadB, GadC, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011-0300588 A1 (SANTOS, CHRISTINE et al.) 08 December 2011 See claims 1, 10 and 28; paragraph [0168]; table 3.3.	13-14
A		1-12,15-30
Y	JENNISON, AMY V. et al., `The acid-resistance pathways of Shigella flexneri 2457T`, Microbiology, August 2007, Vol. 153, No. Pt 8, pp. 2593-2602 See page 2600; abstract.	13-14
A		1-12,15-30
A	WO 2013-087884 A1 (UNIVERSITEIT GENT) 20 June 2013 See claim 17.	1-30
A	MA, ZHUO et al., `GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in Escherichia coli K-12`, Mol. Microbiol., September 2003, Vol. 49, No. 5, pp. 1309-1320 See abstract.	1-30
A	US 2009-0239757 A1 (NIELSEN, JESPER DUUS et al.) 24 September 2009 See claims 10 and 12-14.	1-30

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2014 (17.11.2014)	**18 November 2014 (18.11.2014)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/049933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0300588 A1	08/12/2011	US 8735132 B2	27/05/2014
		WO 2011-140342 A1	10/11/2011
WO 2013-087884 A1	20/06/2013	AU 2012-351501 A1	03/07/2014
		CN 103987841 A	13/08/2014
US 2009-0239757 A1	24/09/2009	CA 2593872 A1	29/06/2006
		EP 1831396 A2	12/09/2007
		WO 2006-066588 A2	29/06/2006
		WO 2006-066588 A3	24/08/2006