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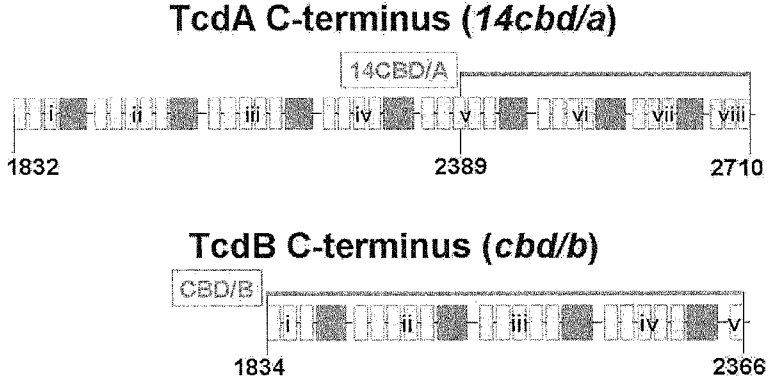
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(54) Title: MULTIVALENT LIVE VECTOR VACCINE AGAINST *CLOSTRIDIUM DIFFICILE-ASSOCIATED DISEASE*

Fig. 1



(57) Abstract: The invention relates to a multivalent *Clostridium difficile* vaccine comprising a *Salmonella* Typhi live vector comprising the cell binding domain of TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof and the cell binding domain of TcdB toxin (CBD/B) of *Clostridium difficile* or an antigenic fragment thereof and optionally the cell-binding subunit component (CdtB) of binary toxin of *Clostridium difficile* or an antigenic fragment thereof. The invention further provides methods of inducing an immune response and methods of preventing recurrence of *C. difficile* infections in subjects.

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Multivalent Live Vector Vaccine against *Clostridium difficile-Associated Disease*

Inventor: James E. Galen

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Appl. No. 61/261,406, filed November 16, 2009. The content of the aforesaid application is relied upon and incorporated by reference in its entirety.

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with government support under Grant No. AI057168 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

Incorporated by reference in its entirety herein is a computer-readable sequence listing submitted concurrently herewith and identified as follows: One 108,528 Byte ASCII (Text) file named "SEQ_Listing.TXT," created on November 16, 2010.

FIELD OF THE INVENTION

The field of the invention relates to vaccines. In particular, the field of the invention relates to therapies to treat and prevent *Clostridium difficile* infections (CDI), including recurrent infections in patients previously infected with *Clostridium difficile.*

BACKGROUND OF THE INVENTION

C. difficile is a rapidly emerging enteric pathogen which is now the leading cause of nosocomial infectious diarrhea in developed countries. Infections are caused by an anaerobic, spore-forming bacillus that often attacks elderly patients in various healthcare settings following treatment of unrelated infections with antibiotics (Blossom DB, McDonald LC. The challenges posed by reemerging *Clostridium difficile* infection. *Clin Infect Dis.* 2007;45:222-227; Gould CV, McDonald LC. Bench-to-bedside review: *Clostridium difficile* colitis. *Crit Care.*

2008;12:203). Clinical symptoms range in severity from mild antibiotic-associated diarrhea to a more severe and life-threatening pseudomembranous colitis which if untreated leads to fulminant colitis and death. (Kelly CP, LaMont JT. *Clostridium difficile* infection. *Annu Rev Med.* 1998;49:375-390). Deaths attributable to *C. difficile* disease have quadrupled in the United States from 5.7 per million persons in 1999 to 23.7 per million in 2004 (Redelings MD, Sorvillo F, Mascola L. Increase in *Clostridium difficile*-related mortality rates, United States, 1999-2004. *Emerg Infect Dis.* 2007;13:1417-1419). Estimates of the cost for treatment for CDI in the United States have been dramatically revised upward from $1 billion in 2002 to $3.2 billion in 2007, due to a dramatic increase in the number of cases and increasing severity of the disease (O'Brien JA, Lahue BJ, Caro JJ, Davidson DM. The emerging infectious challenge of *Clostridium difficile*-associated disease in Massachusetts hospitals: clinical and economic consequences. *Infect Control Hosp Epidemiol.* 2007;28:1219-1227). Rapidly emerging hypervirulent antibiotic-resistant strains of *C. difficile* have been associated with recent epidemics of CDI in North America and Europe with increased morbidity and mortality in healthcare settings (Warny M, Pepin J, Fang A et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet.* 2005;366:1079-1084). However, epidemic strains of *C. difficile* are being increasingly reported in community-acquired disease in which no previous history of antibiotic use was evident. Since epidemic strains of *C. difficile* spores have now been repeatedly detected in commercial beef, poultry, and other food products, increasing attention is now being focused on food products as one possible source of community infection (Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis.* 2007;13:485-487). Although primary CDI can be successfully treated with metronidazole or vancomycin, recurrent episodes of antibiotic-resistant CDI complicate management, and development of a vaccine against infection with *Clostridium difficile* could be useful to prevent relapse (Musher DM, Nuila F, Logan N. The long-term outcome of treatment of *Clostridium difficile* colitis. *Clin Infect Dis.* 2007;45:523-524).

One significant challenge in the management of CDI is successful treatment of recurrent disease after resolution of primary disease symptoms. Recurrent CDI (RCDI) typically occurs within 7 days to 3 weeks following cessation of antibiotic treatment for the initial episode. The most significant risk factor for recurrence is recurrence itself (Blossom DB, McDonald LC. The challenges posed by reemerging *Clostridium difficile* infection. *Clin Infect Dis.* 2007;45:222-227). The risk of recurrent infection rises from about 20% after the primary infection to approximately 40% after the first recurrence, further increasing to >60% after two or more recurrences (Kelly CP, LaMont JT. *Clostridium difficile*--more difficult than ever. *N Engl J Med.* 2008;359:1932-1940). Epidemic strains of *C. difficile*, including PCR ribotypes 027 and 078, are associated with recurrence and increased severity of disease (Leav BA, Blair B, Leney M et al. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). *Vaccine.* 2010;28:965-969; Goorhuis A, Bakker D, Corver J et al. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis.* 2008;47:1162-1170; Burns K, Morris-Downes M, Fawley WN, Smyth E, Wilcox MH, Fitzpatrick F. Infection due to C. difficile ribotype 078: first report of cases in the Republic of Ireland. *J Hosp Infect.* 2010;75:287-291). In one recent study, these two ribotypes accounted for 54% of typeable isolates from recurrent CDI cases, and 34% of primary cases identified over a one month period (Burns K, Skally M, Solomon K et al. *Clostridium difficile* Infection in the Republic of Ireland: Results of a 1-Month National Surveillance and Ribotyping Project, March 2009. *Infect Control Hosp Epidemiol.* 2010). Other risk factors for RCDI include age >65 years, sever underlying illness, and continued use of antibiotics for unrelated disease after resolution of CDI; however, the coincidence of more than one of these factors dramatically increases the probability of recurrence (Kyne L, Warny M, Qamar A, Kelly CP. Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet.* 2001;357:189-193; Hu MY, Katchar K, Kyne L et al. Prospective derivation and validation of a clinical prediction rule for recurrent *Clostridium difficile* infection. *Gastroenterology.* 2009;136:1206-1214).

The genome of *C. difficile* is highly dynamic, and recent studies point to multiple paths leading to the emergence of hypervirulence in epidemic strains (He M, Sebaihia M, Lawley TD *et al.* Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A.* 2010;107:7527-7532). Such genome fluidity suggests that modulation of virulence would be expected, with variation shaped through positive selection by host immunity as well as clinical pressures including routine antibiotic therapy (He M, Sebaihia M, Lawley TD et al. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A.* 2010;107:7527-7532; Stabler RA, He M, Dawson L et al. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biol.* 2009;10:R102; Stabler RA, Valiente E, Dawson LF, He M, Parkhill J, Wren BW. In-depth genetic analysis of *Clostridium difficile* PCR-ribotype 027 strains reveals high genome fluidity including point mutations and inversions. *Gut Microbes.* 2010;1:1-8). Genome fluidity seems to explain the repeated isolation of clinical strains of *C. difficile* in which expected virulence factors are not always present.

Enterotoxins A (TcdA) and B (TcdB) are the primary virulence factors of *C. difficile.* These toxins are exoenzymes that monoglucosylate small Rho-like GTPases, ultimately leading to the disruption of the actin cytoskeleton of colonic intestinal epithelial cells, destruction of tight junctions, and apoptosis (Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev.* 2005;18:247-263; Aktories K, Barbieri JT. Bacterial cytotoxins: targeting eukaryotic switches. *Nat Rev Microbiol.* 2005;3:397-410). Release of cytokines from intoxicated target cells also leads to massive infiltration of neutrophils into damaged tissue regions, a hallmark of pseudomembranous colitis (Thielman Nathan M., Wilson Kenneth H. Antibiotic-Associated Colitis. In: Mandell GL, Bennett John E, Dolin R, eds. *Principles and Practice of Infectious Diseases. 6 ed.* Philadelphia: Elsevier Churchill Livingstone; 2005:1249-63). Both enterotoxins are not required to cause disease, and clinical strains in which TcdA is absent have been repeatedly isolated from patients (Kim SJ, Kim H, Seo Y et al. Molecular characterization of toxin A-negative, toxin B-positive variant strains of

Clostridium difficile isolated in Korea. *Diagn Microbiol Infect Dis.* 2010;67:198-201; Pituch H, Brazier JS, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M. Prevalence and association of PCR ribotypes of *Clostridium difficile* isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLSB) type resistance. *J Med Microbiol.* 2006;55:207-213; Pituch H, van LW, Maquelin K *et al.* Toxin profiles and resistances to macrolides and newer fluoroquinolones as epidemicity determinants of clinical isolates of *Clostridium difficile* from Warsaw, Poland. *J Clin Microbiol.* 2007;45:1607-1610). To date, all clinical isolates of *C. difficile* express TcdB, and TcdB is the only virulence factor suggested to be specifically required for manifestation of disease in humans (Lyras D, O'Connor JR, Howarth PM et al. Toxin B is essential for virulence of *Clostridium difficile. Nature.* 2009). Recent studies suggest that TcdB expressed by epidemic strains is hypertoxic due to an extended range of tissue tropism and increased penetration into the cytoplasm of target cells (Lanis JM, Barua S, Ballard JD. Variations in TcdB Activity and the Hypervirulence of Emerging Strains of *Clostridium difficile. PLoS Pathog.* 2010;6:e1001061). However, epidemic strains of *C. difficile*, including PCR ribotypes 027 and 078, typically express both TcdA and TcdB, suggesting that co-expression of both toxins may play an important role in the severity of disease caused by these epidemic strains.

In addition to toxins A and B, PCR-ribotypes 027 and 078 also invariably carry an additional toxin affecting the actin cytoskeleton called *C. difficile* transferase (Cdt); this toxin has also been called binary toxin because it is composed of a catalytic A subunit and a cell-binding B subunit (Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect Immun.* 1997;65:1402-1407; Rupnik M, Grabnar M, Geric B. Binary toxin producing *Clostridium difficile* strains. *Anaerobe.* 2003;9:289-294). The activity of Cdt causes rearrangement of the actin cytoskeleton of intestinal epithelial cells, disrupting tight junctions and allowing better penetration and binding of toxin B to basolateral receptors, possibly enhancing the virulence of epidemic strains (Carter GP, Rood JI, Lyras D. The role of toxin A and toxin B in *Clostridium difficile*-

associated disease. *Gut Microbes.* 2010;1:58-64). Surprisingly, it was recently discovered that Cdt also appears to enhance colonization of the intestinal tract by inducing microtubule-based protrusions which enhance the adherence of *C. difficile* (Schwan C, Stecher B, Tzivelekidis T *et al. Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog.* 2009;5:e1000626).

Since antibiotic use is a major contributing factor to the occurrence of CDI, a non-antibiotic vaccine-based approach for preventing disease could potentially reduce patient morbidity and mortality due to recurrent infection following cessation of antibiotic treatment. Recent economic computer models strongly indicate that development of a vaccine against infection with *C. difficile* could be cost-effective over a wide range of vaccine efficacies and costs when used to prevent recurrent disease (Lee BY, Popovich MJ, Tian Y et al. The potential value of *Clostridium difficile* vaccine: an economic computer simulation model. *Vaccine.* 2010;28:5245-5253). However, no such vaccine is currently on the market.

Reduced serum IgG antibody responses to *C. difficile* toxin A has been proposed as a risk factor linked to recurrence of infection with *C. difficile* (Kyne L, Warny M, Qamar A, Kelly CP. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med.* 2000;342:390-397; Aboudola S, Kotloff KL, Kyne L et al. *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. *Infect Immun.* 2003;71:1608-1610), and evidence from a recent Phase 2 clinical trial indicates that lower serum concentrations of neutralizing antibody against both TcdA and TcdB are associated with recurrence of CDI (Leav BA, Blair B, Leney M et al. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). *Vaccine.* 2010;28:965-969). Additional data from a related Phase 2 trial showed that co-administration of antibiotics to patients with CDI, along with humanized IgG monoclonal antibodies neutralizing toxin A (CDA1) and toxin B (CDB1), provided significant protection against recurrent disease, with patients suffering from multiple recurrences being particularly likely to benefit (Lowy I, Molrine DC, Leav BA et al. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. *N Engl J Med.* 2010;362:197-205). Sanofi-Aventis

has conducted a series of Phase 1 clinical trials testing the immunogenicity of a 3 dose toxoid-based bivalent vaccine targeting both TcdA and TcdB from *C. difficile*; encouraging serum IgG titers against toxins A and B were observed for both healthy subjects (18-55 years of age) and elderly subjects (≥ 65), with seroconversion rates of 75% against TcdA and TcdB in both age groups after 3 intramuscular 50 μg doses of toxoid (Foglia, G. ACAM-CDIFFTM: An Active Vaccine Against *Clostridium difficile* Infection (CDI). 2010. Ref Type: Conference Proceeding). The mechanism(s) by which serum antibody responses are effective against infection and disease caused by *C. difficile* are unclear, although it has been proposed that entry of IgG antitoxin from the blood into mucosal tissues of the large bowel or intestinal lumen may prevent toxin binding (Kelly CP. Immune response to *Clostridium difficile* infection. *Eur J Gastroenterol Hepatol.* 1996;8:1048-1053; Warny M, Vaerman JP, Avesani V, Delmee M. Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. *Infect Immun.* 1994;62:384-389).

There is a need for new treatments against *C. difficile* infections, in particular, treatments aimed at preventing recurrent infections of *C. difficile* in patients. The present invention of a multivalent live *Salmonella enterica* serovar Typhi (*Salmonella* Typhi; *S.* Typhi) vector expressing various toxins from *Clostridium difficile* satisfies this need.

SUMMARY OF THE INVENTION

The invention provides a *Clostridium difficile* vaccine comprising a live *Salmonella* Typhi vector comprising the cell binding domain of *Clostridium difficile* toxin TcdA (CBD/A) or an antigenic fragment thereof and the cell binding domain of *Clostridium difficile* toxin TcdB (CBD/B) or an antigenic fragment thereof.

In another aspect, the invention further provides a *Clostridium difficile* vaccine comprising a *Salmonella* Typhi vector comprising the cell binding domains of the *Clostridium difficile* toxins TcdA (CBD/A) and TcdB (CBD/B) or antigenic fragments thereof, and additionally comprises the cell-binding subunit

(CdtB) of the *Clostridium difficile* colonization factor binary toxin, or an antigenic fragment thereof.

In another aspect, the invention provides a *Clostridium difficile* vaccine comprising a *Salmonella* Typhi vector wherein domains of one or more of the TcdA (CBD/A) toxin, the TcdB (CBD/B) toxin and CdtB toxin are expressed on a non-antibiotic plasmid selection system in *Salmonella* Typhi. In some embodiments of the vaccine of the invention, the plasmid expresses a gene that is essential for the growth of *Salmonella* Typhi, and has necessarily also been deleted from the *Salmonella* Typhi chromosome. In some embodiments, the gene encodes single stranded binding protein (SSB).

In some aspects, the invention provides a *Clostridium difficile* vaccine comprising a *Salmonella* Typhi vector wherein synthetic genes encoding domains of one or more of the TcdA (CBD/A) toxin, the TcdB (CBD/B) toxin and CdtB toxin are chromosomally integrated into the genome of *Salmonella* Typhi. In some embodiments of the invention, the TcdB (CBD/B) toxin is expressed on a non-antibiotic plasmid expression system in *Salmonella* Typhi, and synthetic genes encoding domains of TcdA (CBD/A) and CdtB toxins are chromosomally integrated into the genome of *Salmonella* Typhi. In some embodiments, one or both synthetic genes encoding domains of TcdA (CBD/A) and CdtB are inserted into the *guaBA* locus of *Salmonella* Typhi. In some embodiments of the invention, synthetic genes encoding domains of either the TcdA (CBD/A) toxin or CdtB binary toxin is inserted into the *guaBA* locus of *S.* Typhi and the remaining gene is inserted into the *htrA* locus of *S.* Typhi.

In another aspect, the invention provides a *Clostridium difficile* vaccine comprising a *Salmonella* Typhi vector wherein domains of one or more of TcdA toxin protein (CBD/A), TcdB toxin protein (CBD/B) and CdtB binary toxin protein are fused to a cytolysin A (ClyA) protein from *Salmonella* Typhi to facilitate export of the toxin from the cell. In some embodiments, ClyA is mutated to reduce hemolytic activity of ClyA, while still retaining the ability to export the toxin from the cell. In one embodiment, the ClyA mutant is ClyA I198N. In another embodiment, the ClyA mutant is ClyA C285W.

In another aspect, the invention provides a *Clostridium difficile* vaccine comprising a *Salmonella* Typhi live vector wherein the nucleic acid sequences encoding non-toxic cell-binding domains of TcdA toxin (CBD/A), TcdB toxin (CBD/B) and CdtB or antigenic fragments thereof are genetically optimized for expression and stability in *Salmonella* Typhi.

In another aspect, the present invention is directed to methods of inducing an immune response against *Clostridium difficile* in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a *Salmonella* Typhi live vector of the invention.

In another aspect, the present invention is directed to methods of inducing an immune response against *Clostridium difficile* in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a *Salmonella* Typhi live vector of the invention as a prime, and subsequently administering a boost comprising a polypeptide selected from the group consisting of TcdA toxin (CBD/A) or an antigenic fragment thereof, TcdB toxin (CBD/B) or an antigenic fragment thereof, and CdtB or an antigenic fragment thereof, or combinations thereof.

In another aspect, the invention is directed to preventing recurrence of a *Clostridium difficile* infection in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a *Salmonella* Typhi live vector of the invention. In another aspect, the present invention is directed to preventing recurrence of a *Clostridium difficile* infection in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a *Salmonella* Typhi live vector of the invention as a prime, and subsequently administering a boost that comprises a polypeptide selected from the group consisting of TcdA toxin (CBD/A) or an antigenic fragment thereof, TcdB toxin (CBD/B) or an antigenic fragment thereof, and CdtB or an antigenic fragment thereof, or combinations thereof.

In some aspects of the invention, the *S.* Typhi live vector is administered mucosally.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Synthetic codon-optimized gene cassettes encoding regions of the cell-binding domains of *C. difficile* toxins A and B. 14CBD/A comprises residues 2389 to 2710 of toxin A and CBD/B comprises residues 1834-2366 of toxin B.

FIG. 2. Western immunoblot of whole cell lystes from attenuated *S.* Typhi CVD 908-*htrAssb* expressing fused and unfused CBD/B antigens from the cell-binding domain of *C. difficile* toxin B.

FIG. 3. Western immunoblot of culture supernatants from DH5α expressing non-hemolytic fusions of ClyA fused to the fluorescent reporter protein GFPuv (ClyA*-GFPuv) or wildtype ClyA-GFPuv protein. (A.) Culture supernatants stained with anti-GFP polyclonal antibody to detect properly exported ClyA*-GFPuv fusions. (B.) Culture supernatants stained with polyclonal antibody against the cytoplasmic protein GroEL; a lysate of CVD 908-*htrA* (pClyA-GFPuv) was included as a control for background autolysis of live vectors.

FIG. 4. Immunogenicity of attenuated *S.* Typhi CVD 908-*htrA* in mice using a heterologous mucosal prime-parenteral boost strategy. Animals were immunized intranasally on days 0 and 14 with 5 x 10^9 colony forming units (CFU) of plasmid-bearing live vectors, and boosted intramuscularly on day 42 with 0.5 μg purified GFPuv adsorbed to 0.5 mg Alhydrogel in a total volume of 50 μl. Mice were immunized with live vectors carrying medium copy number expression plasmids encoding GFPuv fused to the carboxyl terminus of ClyA, in which ClyA also contained either: 1] a single mutation abolishing hemolytic activity (pI198N*gfpuv*); 2] the set of triple mutations I198N,A199D,E204K abolishing hemolytic activity (pSEC93*gfpuv*); or 3] wildtype ClyA (pSEC91*gfpuv*). CVD 908-*htrA* was included as an empty vector control.

FIG. 5. SSB-stabilized medium copy (pSEC91-83S, ~ 15 copies per chromosomal equivalent) or low copy (pSEC10-83S, ~ 5 copies per chromosomal equivalent) plasmids in which PA83 is fused to ClyA.

FIG. 6. (A) Isogenic SSB-stabilized versions of pGEN222 and pGEN206. (B) After electroporating these four isogenic expression plasmids into CVD 908-*htrA*, the desired live vector strains were recovered, and an in vitro plasmid stability

experiment was carried out. Viable counts were recovered on non-selective medium, and the percentage of fluorescing colonies determined.

FIG. 7. A plasmid map of pSEC10S-CBDB, an expression vector for *S.* Typhi encoding a fusion of ClyA and the cell-binding domain fragment of TcdB (CBD/B).

FIG. 8. A plasmid map of pCBDB, an expression vector for *S.* Typhi encoding a cell-binding domain fragment of TcdB (CBD/B).

FIG. 9. Integration of a synthetic codon-optimized gene cassette, encoding a fusion of non-hemolytic ClyA* fused to the cell-binding domain of *C. difficile* toxin A (14CBD/A), integrated into the chromosomal Δ*guaBA* locus of the CVD 910*ssb* attenuated live vector. The blue and yellow ovals denote nested P_{guaB} and P_{ompC} promoters controlling expression of *clyA*-14cbd/A* by growth rate and osmolarity respectively. The intact wildtype chromosomal loci *xseA* and *t0348* from *S.* Typhi strain Ty2, which flank the Δ*guaBA::clyA*-14cbd/a* insertion, are represented by arrows.

FIG. 10. Attenuated *S.* Typhi-based trivalent live vector vaccine against recurrent *C. difficile* infections (RCDI). Genetic fusions encoding the non-hemolytic *clyA** antigen export protein fused to the cell-binding domain of TcdA (*clyA*-14cbd/a*) or TcdB (*clyA*-cbd/b*) are depicted as chromosomal insertions into the deleted *guaBA* and *htrA* loci of CVD 910*ssb* respectively. The remaining CBD/BT antigen of this trivalent vaccine is encoded by the low copy number SSB-stabilized plasmid, pCBD/BT, which also encodes a plasmid inheritance function (*parA*) and the plasmid replication protein *repA*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a multivalent vaccine to treat and/or prevent *Clostridium difficile* infection comprising a *Salmonella* Typhi live vector comprising the cell-binding domain of the TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof and the cell-binding domain of TcdB (CBD/B) toxin of *C. difficile* or an antigenic fragment thereof for eliciting a balanced and robust mucosal as well as serum neutralizing antibody response. In

ıddition to targeting toxins A and B, in some aspects, the invention further ɔrovides a *S.* Typhi live vector that further comprises the cell-binding subunit of ɔinary toxin, CdtB from *C. difficile* or an antigenic fragment thereof, thereby ɜncompassing a trivalent live vector vaccine. Binary toxin may act to enhance the /irulence of epidemic strains carrying all 3 toxins, including ribotypes 027 and)78, by promoting better colonization of *C. difficile* from germinating spores for lelivering higher levels of the toxins while improving penetration and binding of ſcdB. Without being bound by any particular theory as to how the invention vorks, the present invention is believed to encompass targeting live vector-nediated immunity against the actions of toxins A and B at two levels: 1] by ılocking the binding of both toxins through targeting of serum and mucosal mmunity to their cell-binding domains, and 2] reducing the binding of toxin B by naintaining the integrity of intestinal epithelial tissue through mucosal ıeutralization of the binding domain of CdtB. Neutralization of CdtB can also ırovide mucosal immunity against intestinal colonization and recurrent infection. n accordance with the embodiments of the invention, the cell binding domains of 'cdA toxin (CBD/A), TcdB toxin (CBD/B), and/or CdtB can be expressed in *'almonella* Typhi on a plasmid, chromosomally, or some combination of plasmid-ased and chromosomalbased expression systems.

The cell binding domain of TcdA toxin (CBD/A) as described herein ıcludes amino acids 1832-2710 of TcdA from *C. difficile* (see FIG. 1). In some mbodiments, the *Salmonella* Typhi live vector expresses a truncated cell binding omain comprising amino acids 2389-2710. The amino acid sequence of the cell inding domains of TcdA of various *C. difficile* strains is provided by SEQ ID ſOS:15-17.

The cell binding domain of TcdB toxin (CBD/B) as described herein ıcludes amino acids 1834-2366 of TcdB from *C. difficile* (see FIG. 1). The amino ɔid sequence of the cell binding domains of various *C. difficile* strains is provided y SEQ ID NOS:18-19.

The invention further provides synthetic genes encoding the TcdA toxin ɔBD/A) and TcdB toxin (CBD/B) that have been optimized for both genetic ability and efficient expression within the *S.* Typhi live vectors of the invention.

Expression cassettes provided herein for TcdA toxin (CBD/A) and TcdB toxin (CBD/B) encode domains from the carboxyl-terminal receptor-binding region of TcdA and TcdB and include unique internal restriction sites to allow for easy manipulation. In one embodiment, restrictions sites NheI and AvrII span the cassette to facilitate easy cloning of the cassettes into expression vectors or to facilitate chromosomal insertion. These two cassettes comprise the C-terminal 322 residues of TcdA (amino acids 2389-2710 of TcdA) (14CBD/A) and comprise the C-terminal 533 residues of TcdB (CBD/B) (See Fig. 1). The nucleotide sequence of the codon optimized cassette of TcdA (14CBD/A) is SEQ ID NO:1 and the amino acid sequence is SEQ ID NO:2. The nucleotide sequence of the codon optimized cassette of TcdB (CBD/B) is SEQ ID NO:3 and the amino acid sequence is SEQ ID NO:4. Shown in Fig. 7 is the TcdB (CBD/B) cassette inserted into the NheI-AvrII sites of the pSEC10 expression plasmid, creating the genetic fusion gene *clyA-cbdb*.The invention further provides a synthetic gene encoding the cell-binding subunit of binary toxin, CdtB, that has been optimized for both genetic stability and efficient expression within the *S.* Tyhi live vectors of the invention. The nucleotide sequence of the codon optimized gene cassette comprising the cell-binding subunit of binary toxin (CdtB)(encoding amino acids 2-876 of the wild-type sequence) is SEQ ID NO:5 and the amino acid sequence is SEQ ID NO:6.

As used herein the term "polypeptide" is used interchangeably with the term "protein" and is further meant to encompass peptides. Therefore, as used herein, a polypeptide is a polymer of two or more amino acids joined together by peptide linkages.

As used herein the term "antigenic fragment" of a particular protein is a fragment of that protein that is antigenic. For example, an antigenic fragment of TcdA (CBD/A), TcdB (CBD/B) or CdtB polypeptide can include a large fragment that is missing as little as a single amino acid. In a particular embodiment, an antigenic fragment of the polypeptide comprises at least 7 amino acid residues. In some embodiments, an antigenic fragment comprises at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 30 amino acid residues, at least 35 amino acid residues, at least 40 amino acid residues, at least 45 amino acid residues or at least

50 amino acid residues. In another embodiment, an antigenic fragment of TcdA (CBD/A), TcdB (CBD/B) or CdtB polypeptide comprises between 50 and 100 amino acid residues.

In yet another embodiment, an antigenic fragment of TcdA (CBD/A) comprises 100 amino acids or more, but fewer than 879 amino acids. In some embodiments, the antigenic fragment of TcdA (CBD/A) comprises between 150 and 850 amino acids, between 150 and 700 amino acids or between 200 and 500 amino acids. In some embodiments, the antigenic fragment comprises amino acids 5-325 of SEQ ID NO 2, amino acids 10-325 of SEQ ID NO 2, amino acids 20-325 of SEQ ID NO 2, amino acids 40-325 of SEQ ID NO 2, amino acids 75-325 of SEQ ID NO 2, amino acids 150-325 of SEQ ID NO 2, or amino acids 200-325 of SEQ ID NO 2. In some embodiments, the antigenic fragment comprises amino acids 4-324 of SEQ ID NO 2, amino acids 4-300 of SEQ ID NO 2, amino acids 4-275 of SEQ ID NO 2, amino acids 4-250 of SEQ ID NO 2, amino acids 4-200 of SEQ ID NO 2, amino acids 4-150 of SEQ ID NO 2, or amino acids 4-100 of SEQ ID NO 2.

In yet another embodiment, an antigenic fragment of TcdB (CBD/B) comprises 100 amino acids or more, but fewer than 533 amino acids. In some embodiments, the antigenic fragment of TcdB (CBD/B) comprises between 150 and 500 amino acids, between 200 and 400 amino acids or between 250 and 350 amino acids. In some embodiments, the antigenic fragment comprises amino acids 5-536 of SEQ ID NO 4, amino acids 10-536 of SEQ ID NO 4, amino acids 20-536 of SEQ ID NO 4, amino acids 40-536 of SEQ ID NO 4, amino acids 75-536 of SEQ ID NO 4, amino acids 150-536 of SEQ ID NO 4, amino acids 250-536 of SEQ ID NO 4, amino acids 350-536 of SEQ ID NO 4, or amino acids 400-536 of SEQ ID NO 4. In some embodiments, the antigenic fragment comprises amino acids 4-535 of SEQ ID NO 4, amino acids 4-500 of SEQ ID NO 4, amino acids 4-400 of SEQ ID NO 4, amino acids 4-350 of SEQ ID NO 4, amino acids 4-275 of SEQ ID NO 4, amino acids 4-200 of SEQ ID NO 4, or amino acids 4-100 of SEQ ID NO 4.

In yet another embodiment, an antigenic fragment of CdtB comprises 100 amino acids or more, but fewer than 876 amino acids. In some embodiments, the

ıntigenic fragment of CdtB comprises between 150 and 650 amino acids, between 200 and 550 amino acids or between 300 and 450 amino acids. In some embodiments, the antigenic fragment comprises amino acids 5-878 of SEQ ID NO 6, amino acids 10-878 of SEQ ID NO 6, amino acids 20-878 of SEQ ID NO 6, ımino acids 40-878 of SEQ ID NO 6, amino acids 75-878 of SEQ ID NO 6, amino ıcids 150-878 of SEQ ID NO 6, amino acids 250-878 of SEQ ID NO 6, amino ıcids 350-878 of SEQ ID NO 6, amino acids 450-533 of SEQ ID NO 6, amino ıcids 550-878 of SEQ ID NO 6, amino acids 650-878 of SEQ ID NO 6, or amino ıcids 750-878 of SEQ ID NO 6. In some embodiments, the antigenic fragment ·omprises amino acids 4-877 of SEQ ID NO 6, amino acids 4-800 of SEQ ID NO 6, amino acids 4-700 of SEQ ID NO 6, amino acids 4-600 of SEQ ID NO 6, amino .cids 4-500 of SEQ ID NO 6, amino acids 4-450 of SEQ ID NO 6, or amino acids -250 of SEQ ID NO 6.

There is no limitation on how the antigenic fragment can be made. For xample, an antigenic fragment can be obtained from a recombinant source, from a ıotein isolated from natural sources, or through chemical synthesis. Similarly, an ntigenic fragment can be obtained following the proteolytic digestion of such roteins or fragments.

'almonella Typhi Live Vector Vaccines

Salmonella has been adapted for use as an attenuated live oral vaccine to eliver foreign antigens. Several attenuated *S.* Typhi strains have been developed ver the years, including *S.* Typhi CVD 908 (Hone, D. M., A. M. Harris, S. :hatfield, G. Dougan, and M. M. Levine. 1991. Construction of genetically efined double aro mutants of Salmonella typhi. *Vaccine* 9:810-816; Tacket, C. O., M. Sztein, G. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, i. Dougan, S. Chatfield, and M. M. Levine. 1997. Safety of live oral Salmonella /phi vaccine strains with deletions in htrA and aroC aroD and immune responses ı humans. *Infect. Immun.* 65:452-456; Wang, J. Y., F. Noriega, J. E. Galen, E. M. arry, and M. M. Levine. 2000. Constitutive expression of the Vi polysaccharide apsular antigen in attenuated Salmonella enterica serovar Typhi oral vaccine rain CVD 909. *Infect. Immun.* 68:4647-4652; Wang, J. Y., M. F. Pasetti, F.

Noriega, R. J. Anderson, S. S. Wasserman, J. E. Galen, M. Sztein, and M. M. Levine. 2001. Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated DguaBA Salmonella enterica serovar Typhi strain CVD 915. *Infect. Immun.* 69:4734-4741). *S.* Typhi CVD 908 is attenuated through stable chromosomal integration of two non-reverting deletion mutations within the *aroC* and *aroD* genes. These two genes encode enzymes critical in the biosynthesis of several aromatic amino acids, as well as biosynthesis of the purine nucleotides ATP and GTP. The pleiotropic effect of the *ΔaroCΔaroD* double deletion mutations is responsible for the level of attenuation observed when CVD 908 was given to volunteers in Phase 1 studies. Immunization with CVD 908 was quite well tolerated and highly immunogenic (Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine. 1992. Comparison of the safety and immunogenicity of DaroCDaroD and DcyaDcrp *Salmonella typhi* strains in adult volunteers. *Infect. Immun.* 60:536-541). However, a clinically silent primary bacteremia was detected in 100% of volunteers who ingested 5 x 10^8 colony forming units (CFU), wherein vaccine organisms were recovered from blood cultures collected between days 4 and 8 after vaccination (Levine, M. M., J. E. Galen, E. M. Barry, F. Noriega, S. Chatfield, M. Sztein, G. Dougan, and C. O. Tacket. 1996. Attenuated Salmonella as live oral vaccines against typhoid fever and as live vectors. *J. Biotechnol.* 44:193-196.) This silent vaccinemia presumably resulted from migration of the vaccine strain to the organs of the reticuloendothelial system, but was not associated with adverse clinical symptoms and spontaneously resolved without antibiotic intervention.

CVD 908 has been further attenuated to avoid this silent vaccinemia by introducing an additional defined deletion mutation into the *htrA* gene encoding a stress-induced serine protease responsible for degradation of mis-folded periplasmic proteins (Pallen, M. J. and B. W. Wren. 1997. The HtrA family of serine proteases. *Mol. Microbiol.* 26:209-221). The *htrA* locus was chosen because *htrA* mutants in *S.* Typhimurium were less virulent in mice, and were shown *in vitro* to be less able to withstand the oxidative burst following phagocytosis into macrophages (Chatfield, S., K. Strahan, D. Pickard, I. G. Charles, C. E.

Hormaeche, and G. Dougan. 1992. Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. *Microb. Pathog.* 12:145-151; Baumler, A. J., J. G. Kusters, I. Stojiljkovic, and F. Heffron. 1994. *Salmonella typhimurium* loci involved in survival within macrophages. *Infect. Immun.* 62:1623-1630). The resulting CVD 908-*htrA* vaccine strain was found to be well tolerated at doses up to 5 x 10^9 CFU with no positive blood cultures detected. In addition, CVD 908-*htrA* elicited a broad immune response to *S.* Typhi antigens that included intestinal secretory IgA antibodies, serum IgG antibodies, and cellular immune responses (Salerno-Goncalves, R., T. L. Wyant, M. F. Pasetti, M. Fernandez-Vina, C. O. Tacket, M. M. Levine, and M. B. Sztein. 2003. Concomitant induction of CD4+ and CD8+ T cell responses in volunteers immunized with *Salmonella* enterica serovar typhi strain CVD 908-htrA. *J. Immunol.* 170:2734-2741; Tacket, C. O., M. Sztein, G. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. Chatfield, and M. M. Levine. 1997. Safety of live oral *Salmonella* typhi vaccine strains with deletions in htrA and aroC aroD and immune responses in humans. *Infect. Immun.* 65:452-456; Tacket, C. O., M. Sztein, S. S. Wasserman, G. Losonsky, K. Kotloff, T. L. Wyant, J. P. Nataro, R. Edelman, J. G. Perry, P. Bedford, D. Brown, S. Chatfield, G. Dougan, and M. M. Levine. 2000. Phase 2 clinical trial of attenuated *Salmonella* enterica serovar Typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers. *Infect. Immun.* 68:1196-1201). *S.* Typhi is characterized by enteric routes of infection, a quality which permits oral vaccine delivery. *S.* Typhi also infects monocytes and macrophages and can therefore target antigens to professional antigen presenting cells (APCs).

The *Salmonella* Typhi strain that can be used in the present invention as a vaccine is not limited to any particular strain that has been genetically attenuated from the original clinical isolate Ty2. Any attenuated *Salmonella* Typhi strain derived from Ty2 can be used as a live vector in accordance with the invention. Non-limiting, exemplary attenuated *Salmonella* Typhi strains include *S.* Typhi Ty21a, CVD 908, *S.* Typhi CVD 909, CVD 908-*htrA*, and CVD 915. In some embodiments, the *S.* Typhi strain can carry one or more additional chromosomal mutations in an essential gene that is expressed on a plasmid which also encodes a

C. difficile toxin, in accordance with the invention, enabling selection and stabilization of the plasmid and preventing loss in *S.* Typhi. In some embodiments, the *S.* Typhi strain carries a mutation in the *ssb* gene which is encoded on a selection expression plasmid. In some embodiments, the *S.* Typhi strain carrying a *ssb* mutation is selected from *S.* Typhi CVD 908*ssb* and CVD 908-*htrAssb*.

One or more of the TcdA toxin (CBD/A), TcdB (CBD/B) toxin and CdtB toxin proteins can be expressed on one or more plasmids in the live *S.* Typhi vector of the invention. In some embodiments, only one toxin is expressed on a plasmid, while the one or more genes encoding the remaining toxins of *C. difficile* are chromosomally inserted and expressed. In some embodiments, at least two toxin genes are expressed on a plasmid.

Plasmid stability is a key factor in the development of high quality attenuated *S.* Typhi vaccines with the ability to consistently express foreign antigens. Plasmidless bacterial cells tend to accumulate more rapidly than plasmid-bearing cells. One reason for this increased rate of accumulation is that the transcription and translation of plasmid genes imposes a metabolic burden which slows cell growth and gives plasmidless cells a competitive advantage. Furthermore, foreign plasmid gene products are sometimes toxic to the host cell. Thus, it is advantageous for the plasmid to be under some form of selective pressure, in order to ensure that the encoded antigens are properly and efficiently expressed, so that a robust and effective immune response can be achieved.

In some embodiments, the plasmid is selected within *S.* Typhi using a non-antibiotic selection system. For example, the plasmid can encode an essential gene that complements an otherwise lethal deletion/mutation of this locus from the live vector chromosome. Exemplary non-antibiotic expression plasmids that can be used in the invention are described herein and further plasmid systems which can be used in the invention are described, for example, in U.S. Patent Appl. Pub. No. 20070281348, U.S. Pat. Nos. 7,141,408, 7,138,112, 7,125,720, 6,977,176, 6,969,513, 6,703,233, and 6,413,768, which are herein incorporated by reference.

The present inventor has recently developeda non-antibiotic genetic stabilization and selection system for expression plasmids engineered to encode single-stranded binding protein (SSB), an essential protein involved in DNA

replication, recombination, and repair which is deleted from the *S.* Typhi live vector chromosome (Lohman TM, Ferrari ME. Escherichia coli single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annu Rev Biochem.* 1994;63:527-570; Chase JW, Williams KR. Single-stranded DNA binding proteins required for DNA replication. *Annu Rev Biochem.* 1986;55:103-136; Galen JE, Wang JY, Chinchilla M, Vindurampulle C, Vogel JE, Levy H, Blackwelder WC, Pasetti MF, Levine MM. A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in *Salmonella enterica* serovar Typhi live vectors. *Infect Immun.* 2010 Jan;78(1):337-47). In some embodiments, the plasmid expression vector for *S.* Typhi encodes a single-stranded binding protein (SSB). In some embodiments, the expression vector is pSEC10S as described herein.

In some embodiments of the invention, expression plasmids are employed in which both the random segregation and catalytic limitations inherent in non-antibiotic plasmid selection systems have been removed. The segregation of these plasmids within *S.* Typhi live vectors is improved using an active partitioning system (*parA*) for *S.* Typhi CVD 908-*htrA* (Galen, J. E., J. Nair, J. Y. Wang, S. S. Wasserman, M. K. Tanner, M. Sztein, and M. M. Levine. 1999. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella* typhi CVD 908-htrA. *Infect. Immun.* 67:6424-6433). In some embodiments, dependence on catalytic enzymes is avoided by using a plasmid selection/post-segregational killing system based on the *ssb* gene.

Chromosomal Expression of C. difficile toxins

A solution to the instability of multicopy plasmids and the foreign antigens they encode is to integrate foreign gene cassettes into the chromosome of the live vector. . However, the drop in copy number becomes both an advantage and a disadvantage; while the reduced copy number will certainly reduce the metabolic burden associated with both the multicopy plasmid itself and the encoded foreign protein(s), this reduction in foreign antigen synthesis ultimately leads to reduced delivery of these antigens to the host immune system and possibly reduced immunogenicity. This explanation could account for why in clinical trials serum

immune responses to chromosomally encoded antigens have to date been modest. (Gonzalez C, Hone D, Noriega FR *et al. Salmonella* typhi vaccine strain CVD 908 expressing the circumsporozoite protein of Plasmodium falciparum: strain construction and safety and immunogenicity in humans. *J Infect Dis.* 1994;169:927-931; Khan S, Chatfield S, Stratford R et al. Ability of SPI2 mutant of S. typhi to effectively induce antibody responses to the mucosal antigen enterotoxigenic E. coli heat labile toxin B subunit after oral delivery to humans. *Vaccine.* 2007;25:4175-4182). To date the only chromosomal locus used in clinical trials for expression of foreign antigens is aroC.

The present invention provides for chromosomal insertion of one or more *C. difficile* toxin cassettes into the *guaBA* locus and/or *htrA* locus of *S.* Typhi. It will be appreciated that inserting the gene cassettes into either the *guaBA* locus or the *htrA* locus of *S.* Typhi is routine, and can be accomplished, for example, using standard techniques in molecular biology, including the lambda Red recombination system (Datsenko KA and Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *PNAS.* 2000. 97(12): 6640-5.).

Immunogenic cassettes can be integrated into either the Δ*guaBA* or Δ*htrA* locus of CVD 910*ssb*, for example, to compare the immunogenicity of chromosomal integrations versus antigen-specific immunogenicity elicited by plasmid-based expression. In some embodiments, only the open reading frames of Δ*guaBA* and Δ*htrA* are deleted, leaving the original promoters for these sites intact. In some embodiments, insertion cassettes include the P_{ompC} promoter from the low copy expression plasmids, such that integration into Δ*guaBA* or Δ*htrA* results in nested promoters controlling inducible expression of a given cassette at two levels. For example, integration of a P_{ompC}-*clyA*-14cbd/a* cassette (encoding the ClyA*-14CBD/A fusion) into Δ*guaBA* of CVD 910*ssb* would result in inducible transcription of *clyA*-14cbd/a* with both growth rate and osmolarity, as shown in FIG. 9. A synthetic gene encoding the TcdA toxin (CBD/A) or an antigenic fragment thereof can be inserted into *guaBA* locus, the *htrA* locus, or both loci. A synthetic gene encoding the TcdB toxin (CBD/B) or an antigenic fragment thereof can be inserted into *guaBA* locus, the *htrA* locus, or both loci. A synthetic gene

ecoding CdtB or an antigenic fragment thereof can be inserted into *guaBA* locus, the *htrA* locus, or both loci. In one embodiment, the invention provides a *Salmonella* Typhi live vector vaccine wherein CdtB is inserted into the *htrA* locus, TcdA toxin (CBD/A) is inserted into the *guaBA* locus and the TcdB toxin (CBD/B) is expressed on a plasmid.

Export of TcdA (CBD/A), TcdB (CBD/B) and CdtB out of S. Typhi *live vectors using ClyA fusions*

The invention also provides for the use of an extracellular antigen export system derived from a cryptic hemolysin encoded by *clyA* within the chromosome of CVD 908-*htrA* (Galen, J. E., L. Zhao, M. Chinchilla, J. Y. Wang, M. F. Pasetti, J. Green, and M. M. Levine. 2004. Adaptation of the endogenous *Salmonella* enterica serovar Typhi clyA-encoded hemolysin for antigen export enhances the immunogenicity of anthrax protective antigen domain 4 expressed by the attenuated live-vector vaccine strain CVD 908-htrA. *Infect. Immun.* 72:7096-7106). In some embodiments, one or more of TcdA (CBD/A), CdtB and TcdB (CBD/B) are fused to either wild-type or mutant cytolysin A (ClyA) protein from *S.* Typhi to facilitate export from the cell and contact with antibodies to thereby enhance immunogenicity. The ClyA export system is described in U.S. Patent No. 7,459,161, which is incorporated by reference herein.

The ClyA protein is exported from *S.* Typhi and is capable of exporting passenger proteins that have been genetically fused to the 3'-terminus of the *clyA* open reading frame. The nucleotide and amino acid sequence for the isolated *clyA* gene and ClyA protein useful in accordance with the invention are provided as SEQ ID NO:13 and SEQ ID NO:14, respectively. Cytolysin A (ClyA) from *S.* Typhi was first described by Wallace *et al.*, who also reported the crystal structure for the homologous HlyE hemolysin from *E. coli.* (Wallace, A. J., T. J. Stillman, A. Atkins, S. J. Jamieson, P. A. Bullough, J. Green, and P. J. Artymiuk. 2000. E. coli hemolysin E (HlyE, ClyA, SheA): X-ray crystal structure of the toxin and observation of membrane pores by electron microscopy. *Cell* 100:265-276.) HlyE is a kinked rod-shaped 35 kDa molecule with a hydrophobic 27 residue transmembrane region comprising one terminus of the folded molecule. HlyE is

exported into the surrounding medium, but is not exported by any of the known secretion pathways. This hemolysin has been described previously and variously referred to as ClyA, HlyE, or SheA. To avoid confusion, the *E. coli* hemolysin is referred to herein as HlyE and is encoded by hlyE. Also for clarity, the *S.* Typhi hemolysin is referred to herein as ClyA, which is encoded by *clyA*.

ClyA protein typically causes hemolysis in target cells. The present invention encompasses use of both hemolytically active and hemolytically inactive forms of ClyA, with hemolytically inactive mutant forms being more preferred where preservation of antigen export and immunogenicity of the resulting protein fusions can be maintained. In some embodiments, the ClyA is mutated to reduce the hemolytic activity of ClyA while still retaining the export function of ClyA. In one embodiment, the ClyA mutant is ClyA I198N. In another embodiment, the ClyA mutant is ClyA C285W.

Pharmaceutical Formulations

It is contemplated that the *S.* Typhi live vector vaccines of the present invention will be administered in pharmaceutical formulations for use in vaccination of individuals, preferably humans. Such pharmaceutical formulations may include pharmaceutically effective carriers, and optionally, may include other therapeutic ingredients, such as various adjuvants known in the art.

The carrier or carriers must be pharmaceutically acceptable in the sense that they are compatible with the therapeutic ingredients and are not unduly deleterious to the recipient thereof. The therapeutic ingredient or ingredients are provided in an amount and frequency necessary to achieve the desired immunological effect.

The mode of administration and dosage forms will affect the therapeutic amounts of the compounds which are desirable and efficacious for the vaccination application. The bacterial live vector materials are delivered in an amount capable of eliciting an immune reaction in which it is effective to increase the patient's immune response to the expressed toxin(s).

Delivery modes may include, without limitation, parenteral administration methods, such as subcutaneous (SC) injection, transdermal, intramuscular (IM), intradermal (ID), as well as non-parenteral, e.g., oral, nasal, intravaginal,

pulmonary, opthalmic and/or rectal administration. Oral administration of the live vector vaccine is preferred, while parenteral routes of administration are preferred for peptide based vaccine compositions.

The dose rate and suitable dosage forms for the bacterial live vector vaccine compositions of the present invention may be readily determined by those of ordinary skill in the art without undue experimentation, by use of conventional antibody titer determination techniques and conventional bioefficacy/biocompatibility protocols. Among other things, the dose rate and suitable dosage forms depend on the particular antigen employed, the desired therapeutic effect, and the desired time span of bioactivity.

The bacterial live vector vaccines of the present invention may be usefully administered to the host animal with any other suitable pharmacologically or physiologically active agents, e.g., antigenic and/or other biologically active substances.

Formulations of the present invention can be presented, for example, as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the live *S.* Typhi vector or as a suspension.

Therapeutic Methods

The present invention also includes methods of inducing an immune response in a subject. The immune response may be directed to one or more *Clostridium difficile* antigens expressed by the *Salmonella* Typhi live vector comprising toxin TcdA (CBD/A) or an antigenic fragment thereof, toxin TcdB (CBD/B) or an antigenic fragment thereof, and the colonization factor CdtB or an antigenic fragment thereof.

In another aspect, the present invention is directed to methods of inducing an immune response against *Clostridium difficile* in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *Salmonella* Typhi vector as described herein. In some embodiments, the live vector is administered mucosally.

Vaccine strategies are well known in the art and therefore the vaccination strategy encompassed by the invention does not limit the invention in any manner.

In certain aspects of the invention, the *S.* Typhi live vector vaccine expressing the *C. difficile* toxin antigens is administered alone in a single application or administered in sequential applications, spaced out over time.

In other aspects of the invention, the *S.* Typhi live vector vaccine is administered as a component of a heterologous prime/boost regimen. "Heterologous prime/boost" strategies are 2-phase immunization regimes involving sequential administration (in a priming phase and a boosting phase) of the same antigen in two different vaccine formulations by the same or different route. In particular aspects of the invention drawn to heterologous prime/boost regimens, a mucosal prime/parenteral boost immunization strategy is used. For example, one or more *S.* Typhi live vector vaccines as taught herein is administered orally and subsequently boosted parentally with a peptide vaccine comprising one or more of TcdA toxin (CBD/A) or an antigenic fragment thereof, toxin TcdB (CBD/B) or an antigenic fragment thereof, and the colonization factor CdtB or an antigenic fragment thereof, or combinations thereof.

In another aspect, the present invention is directed to methods of inducing an immune response against *Clostridium difficile* in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *Salmonella* Typhi vector of the invention as a prime, and subsequently administering a boost composition comprising a polypeptide selected from the group consisting of TcdA toxin (CBD/A) or an antigenic fragment thereof, TcdB toxin (CBD/B) or an antigenic fragment thereof, and CdtB or an antigenic fragment thereof, and combinations thereof.

In another aspect, the invention is directed to preventing recurrence of a *Clostridium difficile* infection in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *S.* Typhi vector as described herein. In another aspect, the present invention is directed to preventing recurrence of a *Clostridium difficile* infection in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *S.* Typhi vector as described herein as a prime, and subsequently administering a boost composition that comprises a polypeptide selected from the group consisting of TcdA toxin (CBD/A) or an antigenic fragment thereof, TcdB

toxin (CBD/B) or an antigenic fragment thereof, and CdtB or an antigenic fragment thereof, and combinations thereof.

In one embodiment, the method of inducing an immune response comprises mucosally administering to the subject in need thereof an immunologically-effective amount of a live *S.* Typhi vector comprising the cell binding domain of toxin TcdA (CBD/A) or an antigenic fragment thereof and the cell binding domain of toxin TcdB (CBD/B) or an antigenic fragment thereof of *Clostridium difficile*, and optionally, further comprising the colonization factor CdtB of *Clostridium difficile* or an antigenic fragment thereof.

In a further embodiment, the method of inducing an immune response comprises administering a *S.* Typhi live vector vaccine of the present invention to a subject in an amount sufficient to induce an immune response in the subject (an immunologically-effective amount).

As used herein, an "immune response" is the physiological response of the subject's immune system to an immunizing composition. An immune response may include an innate immune response, an adaptive immune response, or both. In one embodiment of the present invention, the immune response is a protective immune response. A protective immune response confers immunological cellular memory upon the subject, with the effect that a secondary exposure to the same or a similar antigen is characterized by one or more of the following characteristics: shorter lag phase than the lag phase resulting from exposure to the selected antigen in the absence of prior exposure to the immunizing composition; production of antibody which continues for a longer period than production of antibody resulting from exposure to the selected antigen in the absence of prior exposure to the immunizing composition; a change in the type and quality of antibody produced in comparison to the type and quality of antibody produced upon exposure to the selected antigen in the absence of prior exposure to the immunizing composition; a shift in class response, with IgG antibodies appearing in higher concentrations and with greater persistence than IgM, than occurs in response to exposure to the selected antigen in the absence of prior exposure to the immunizing composition; an increased average affinity (binding constant) of the antibodies for the antigen in comparison with the average affinity of antibodies for the antigen resulting from

exposure to the selected antigen in the absence of prior exposure to the immunizing composition; and/or other characteristics known in the art to characterize a secondary immune response.

In a further embodiment, the method of inducing an immune response comprises administering a pharmaceutical formulation comprising one or more *Salmonella* Typhi live vectors of the present invention to a subject in an amount sufficient to induce an immune response in the subject (an immunologically-effective amount). In some embodiments, the immune response is sufficient to confer protective immunity upon the subject against a later *Clostridium difficile* infection.

In another aspect, the invention is directed to methods of inducing an immune response, comprising administering to a subject in need thereof a first vaccine comprising an immunologically-effective amount of a *S.* Typhi live vector expressing at least TcdA toxin (CBD/A), and TcdB toxin (CBD/B) of *Clostridium difficile*, and optionally CtdB or an antigenic fragment thereof, and subsequently a second vaccine comprising an immunologically-effective amount of a peptide vaccine comprising one or more of TcdA toxin (CBD/A) or an antigenic fragment thereof, and TcdB toxin (CBD/B), and CdtB or an antigenic fragment thereof. In one embodiment, a subject is orally administered one or more *S.* Typhi live vectors of the invention, followed by a booster immunization administered parenterally, comprising an immunologically-effective amount of a peptide based vaccine comprising one or more of TcdA toxin (CBD/A) or an antigenic fragment thereof, and TcdB toxin (CBD/B), and CdtB or an antigenic fragment thereof. In some embodiments, the time interval between the first and second vaccinations is 1-2 weeks, 3-4 weeks, 5-6 weeks, 7-9 weeks, 10-12 weeks, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 1.5 years, 2 years, 5 years, 7 years, 10 years and 15 years.

In some embodiments, one or more *S.* Typhi live vector vaccines of the invention are mucosally administered in a first priming administration, followed, optionally, by a second (or third, fourth, fifth, etc. ...) priming administration of the live vector vaccine from about 2 to about 10 weeks later. In some embodiments, a boosting composition is administered from about 3 to about 12

weeks after the priming administration. In some embodiments, the boosting composition is administered from about 3 to about 6 weeks after the priming administration.

In some embodiments, the vaccine of the invention is administered with a pharmaceutically acceptable carrier, such that it provides host immunity against an infection.

The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The characteristics of the carrier depend on the nature of the vaccine and the route of administration. Physiologically and pharmaceutically-acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art. The term "pharmaceutically acceptable" is used to refer to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism.

In practicing immunization protocols for treatment and/or prevention, an immunologically-effective amount of a live *Salmonella* Typhi vector is administered to a subject. As used herein, the term "immunologically-effective amount" means the total amount of a live *S.* Typhi vector that is sufficient to show an enhanced immune response in the subject. When "immunologically-effective amount" is applied to an individual therapeutic agent administered alone, the term refers to that therapeutic agent alone. When applied to a combination, the term refers to combined amounts of the ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

The particular dosage depends upon the age, weight, sex and medical condition of the subject to be treated, as well as on the method of administration. Suitable doses can be readily determined by those of skill in the art.

For peptide based vaccines used for boosting, peptide vaccines can be administered by either single or multiple dosages of an effective amount. In some embodiments, an effective amount of the compositions of the invention can vary from 0.01-200 μg/kg per dose. In other embodiments, an effective amount of the composition of the invention can vary from 0.1-100 μg/kg per dose. Greater doses may be administered on the basis of body weight. The exact dosage can be

determined by routine dose/response protocols known to one of ordinary skill in the art. DNA-based vaccines encoding the peptides can also be used as a boost. The boost can also comprise a combination of peptide and DNA-based vaccines.

In some embodiments, the amount of the peptide based vaccine that is effective for boosting is from about 1 μg or less to about 100 μg or more. In some embodiments, it is from about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45 or 50 μg to about 55, 60, 65, 70, 75, 80, 85, 90, or 95 μg per kg body weight.

The term "subject" as used herein, refers to animals, such as mammals. For example, mammals contemplated include humans, primates, dogs, cats, sheep, cattle, goats, pigs, horses, mice, rats, rabbits, guinea pigs, and the like. The terms "subject," "patient," and "host" are used interchangeably.

In some embodiments, the subject to be administered the *S.* Typhi live vector of the invention presents with an existing *C. difficile* infection, in some cases by showing that either toxin A or B is positively identified in the stool. In such patients who are actively colonized with *C. difficile*, it is expected that they will be already immunologically primed against toxins A and B such that oral immunization with the *S.* Typhi live vector vaccine will elicit an anamnestic neutralizing anti-toxin response against both toxins, as well as binary toxin mucosal immunity, reducing the probability of later recurrent infection. In some embodiments, the subject is administered the vaccine while they are concurrently administered antibiotics to treat the infection, such as vancomycin.

In some embodiments, the subject to be administered the vaccine has been recently hospitalized and/or received antibiotic therapy. The leading risk factors associated with CDAD include recent antibiotic therapy and hospitalization, with attack rates peaking in elderly patients. In some embodiments, the patient is administered the live vector vaccine following a course of antibiotics or shortly following a hospital stay. For example, the subject can be administered the live vector vaccine one week following the course of antibiotics, to allow for clearance of antibiotics and improved colonization of the gastrointestinal tract by the attenuated live *S.* Typhi bacterial vaccine. Improved colonization with *S.* Typhi live vectors will result in delivery of high levels of *C. difficile* antigens to the mucosal immune system to enhance protection against recurrence of infection. In

such patients, the methods are intended to confer protective immunity in the subject against later recurrent *Clostridium difficile* infection(s).

In some embodiments, a human subject between the ages of 50 and 64 years of age is administered the live vector vaccine mucosally, and is boosted at a later time, either with the live vector vaccine or a peptide toxoid preparation. For example, the subsequent boosting can take place when the levels of antibody caused by the original administration begin to decline or fade. This can occur after about 5-10 years in some cases. In some embodiments, a boost can be administered about 5-10 years after the initial priming. In some embodiments, it can be advantageous to administer the boost before the subject turns 65 years old, when patients, in general, start to become refractory to vaccine administrations.

The live *S.* Typhi vectors of the present invention may confer resistance to *Clostridium difficile* by either passive immunization or active immunization. In one embodiment of passive immunization, the vaccine is provided to a subject (e.g. a human or mammal) volunteer, and the elicited antisera is recovered and directly provided to a recipient suspected of having an infection caused by *Clostridium difficile.*

In some embodiments, the present invention provides a means for preventing or attenuating infection by *Clostridium difficile.* As used herein, a vaccine is said to prevent or attenuate a disease if its administration to an individual results either in the total or partial attenuation (i.e. suppression) of a symptom or condition of the disease, or in the total or partial immunity of the individual to the disease.

The invention also provides a method for inducing an immune response which comprises administering to an individual suspected of being at risk for infection caused by *Clostridium difficile* an immunologically-effective amount of an antisera elicited from the exposure of a second individual to a live *S.* Typhi vector of the invention, such that it provides host immunity to the infection.

The live *Salmonella* Typhi vectors of the invention may be administered to warm-blooded mammals of any age. The live *Salmonella* Typhi vectors can be administered as a single or multiple priming doses, followed by one or more boosters. For example, a subject can receive a single dose, then be administered a

booster dose up to 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 9 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, or 10 or more years later. In some embodiments, two booster injections of about 10 μg, 30 μg, 60 μg, 90 μg, 60 μg, 90 μg, 120 μg, 150 μg, 180 μg, 210 μg, 240 μg, 270 μg or about 300 μg are administered at about 2 and again about 13 months after the initial injection. Alternatively, three booster injections are given at 2, 4 and 16 months after the initial injection.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate some embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Example 1. Expression of TcdA and TcdB in attenuated *S.* Typhi live vectors.

Two synthetic codon-optimized genes have been engineered for efficient expression in attenuated *S.* Typhi live vectors. Both cassettes encode domains from the carboxyl-terminal receptor-binding region of TcdA and TcdB and include unique internal restriction sites to allow truncation should expression of the full-length cassettes prove toxic. These two cassettes encode the C-terminal 322 residues of TcdA (14CBD/A) and 533 residues of TcdB (CBD/B) (See Fig. 1). The nucleotide sequence of the codon optimized cassette of CBD/A is SEQ ID NO:1 and the amino acid sequence is SEQ ID NO:2. The nucleotide sequence of the codon optimized cassette of CBD/B is SEQ ID NO:3 and the amino acid sequence is SEQ ID NO:4.

Based on recent work from Greco et al, 14CBD/A would be expected to contain at least 3 carbohydrate receptor-binding sites (Greco A, Ho JG, Lin SJ, Palcic MM, Rupnik M, Ng KK. Carbohydrate recognition by *Clostridium difficile* toxin A. *Nat Struct Mol Biol.* 2006;13:460-461). The entire C-terminal region of

TcdB was elected to be expressed because no structural data has specifically defined any receptor-binding site(s) for TcdB.

Both synthetic genes were engineered with codon usage optimized for efficient expression in *S.* Typhi. Since both cassettes encode highly repetitive regions of the *C. difficile* toxins, the DNA sequences were also engineered to enhance genetic stability by removing inverted repeats and palindromes, and minimizing direct repeats to less than 12 bases in length with theoretical melting temperatures $\leq$ 39 °C. Previous work by Ward et al. has show that expression of a cassette similar to 14CBD/A fused to the carboxyl-terminus of tetanus toxin fragment C and delivered by an auxotrophic strain of *S.* Typhimurium (BRD509), elicited serum neutralizing antibody responses against toxin A in mice immunized intranasally with two doses of 10^7 cfu of live vector followed by boosting with purified 14CDTA. (Ward SJ, Douce G, Figueiredo D, Dougan G, Wren BW. Immunogenicity of a *Salmonella* Typhimurium *aroA aroD* vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. *Infect Immun.* 1999;67:2145-2152).

Both synthetic genes were designed as Nhe I – Avr II cassettes for insertion into expression plasmids either as fusions to *clyA* (to allow antigen export out of live vectors) or as unfused genes (for cytoplasmic expression). Of note, fusion of the C-terminal domain of a much larger region of C-terminal TcdA (720 residues, ~ 6 carbohydrate binding sites) to the *E. coli* hemolysin A secretion system was previously hypothesized to improve immunogenicity by allowing export of this domain out of an attenuated *Vibrio cholerae* live vector. (Ryan ET, Butterton JR, Smith RN, Carroll PA, Crean TI, Calderwood SB. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. *Infect Immun.* 1997;65:2941-2949). The cassettes did not insert into higher copy number (~15 copies per chromosomal equivalent) SSB-stabilized expression plasmids, but both cassettes were readily inserted, in both fused and unfused configurations, into an SSB-encoding low copy (~5 copies) replicon. Desired constructs were confirmed by DNA sequence analysis, and are designated here as pSEC10S-14CBD/A (expressing a truncated Cell Binding

Domain of TcdA fused to ClyA), p14CBD/A (expressing unfused TcdA antigen), pSEC10S-CBD/B (expressing the full-length Cell Binding Domain of TcdB fused to ClyA), and pCBD/B (expressing unfused TcdB antigen). These four constructs were electroporated into CVD 908-*htrAssb* and examined using western immunoblots whole cell lysates for expression of fused and unfused 14CBD/A and CBD/B. As shown in Figure 2, excellent expression of both unfused antigens and antigens fused to ClyA was observed; other experiments confirmed equivalent expression of these *C. difficile* antigens in the less attenuated isogenic parental strain of CVD 908-*htrAssb* called CVD 908*ssb* (data not shown).

An initial experiment was conducted in mice, vaccinating only with live vectors, to determine the immunogenicity of ClyA-CBD/B versus unfused CBD/B in both CVD 908-*htrAssb* and less attenuated parent strain CVD 908*ssb* live vectors. In this experiment, the immunogenicity of a non-hemolytic double mutant (DM) version of ClyA was also tested, in which the substitutions I198N and C285W were both introduced into the wildtype sequence, expressed by the pSEC10S2-DM expression plasmid. BALB/c mice were randomly assorted into 5 groups of 10 mice, and immunized on days 0, 14, and 28 with 5×10^9 colony forming units (CFU) of live vector. Sera were collected on days 0, 13, 27, 42, and 49. ELISA titers (EU/ml) against purified toxin B are shown in table 1 below. These initial results are highly promising and strongly suggest that 1] the less attenuated CVD 908*ssb* elicits higher anti-CBD/B titers than the highly attenuated CVD 908-*htrAssb* live vector, and 2] the immunogenicity of CBD/B fused to ClyA may be superior to unfused CBD/B, possibly due to cytoplasmic degradation of CBD/B as suggested in western data in Figure 2, lane 5; breakdown of unfused CBD/A by western analysis was not observed (data not shown). The nucleotide sequences of the entire plasmids or the amino acids of the encoded antigens as shown in Table 1, below, are as follows:

pSEC10S-CBD/B nucleotide (SEQ ID NO:7); pSEC10S-CBD/B polypeptide (SEQ ID NO:8); pSEC10S2-DM-CBD/B nucleotide (SEQ ID NO:9);pSEC10S2-DM-CBD/B polypeptide (SEQ ID NO:10); pCBD/B nucleotide (SEQ ID NO: 11); pCBD/B polypeptide (SEQ ID NO: 12). In the nomenclature scheme, plasmids

with an "S" are SSB-stabilized versions of the original plasmids (i.e. pSEC10S is SSB-stabilized pSEC10). Therefore, pSEC10S2-DM is pSEC10 carrying SSB, and also carrying the double mutant (DM) of ClyA (pSEC10S2-DM).

Anti-TcdB IgG responses in mice immunized intranasally with attenuated *S.* Typhi live vectors SSB-stabilized low-copy-number expression plasmids.

Strain	Day 0	Day 13 (after 1st dose)	Day 27 (after 2nd dose)	Day 42 (after 3rd dose)	Day 49 (3 wk after 3rd dose)
PBS	12.5	14.4	14.4	21.0	14.9 [A]
908-*htrAssb*(pSEC10S-CBD/B)	12.5	35.3	296.4	1676.4	1618.3 [B]
908*ssb*(pSEC10S-CBD/B)	13.4	25.8	284.9	4591.3	5368.5 [C]
908-*htrAssb*(pCBD/B)	12.5	12.5	12.5	14.4	14.4 [D]
908*ssb*(pCBD/B)	14.4	15.4	36.0	154.4	220.3 [E]
908-*htrAssb*(pSEC10S2-DM-CBD/B)	13.4	13.4	13.4	17.5	19.7
908*ssb*(pSEC10S2-DM-CBD/B)	12.5	12.5	12.5	15.8	14.8

9, the two-sided *p*-values from Student *t* –tests on the logs of antitoxin values are: [C] vs [B] = 0.78; [E] vs [D] = vs [D] = 0.0001; [B] vs [E] = 0.018; [C] vs [D] = 0.0001.

Quantitative hemolytic assays were performed on constructs expressing ClyA-CBD fusions, and results are summarized in Table 2 (Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier. 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect. Immun.* 51:461-469). As expected, fusion of the 36.3 kDa 14CBD/A passenger domain to ClyA reduces hemolytic activity to ~50% of unfused ClyA expressed by pSEC10. Surprisingly, fusion of the larger 61.6 kDa domain of CDB/B to ClyA only reduces hemolytic activity slightly. Although the apparent export of ClyA fusions, resulting in hemolysis of RBCs, suggests successful export of ClyA fusions out of our live vectors, we recognize that introduction of non-hemolytic mutations into *clyA* that still preserve export will be required for clinical acceptability.

Table 2. Hemolytic activity of CVD 908-*htrA* expressing protein fusions of ClyA fused to 14CBD/A or CDB/B.

Strain	Hemolytic activity (OD_{545})
htrA	**0.028**
***htrA*(pSEC10)**	**1.231**
***htrA*(pSEC10-14CBD/A)**	**0.583**
***htrA*(pSEC10-CBD/B)**	**1.151**
Ty21a	**0.106**

Example 2. Molecular evolution of non-hemolytic alleles of *clyA*.

ClyA was phenotypically identified in *Salmonella* strains as a cryptic hemolysin, present in several serovars including the licensed *S.* Typhi vaccine strain Ty21a (Oscarsson J, Westermark M, Lofdahl S et al. Characterization of a pore-forming cytotoxin expressed by *Salmonella enterica* serovars typhi and paratyphi A. *Infect Immun.* 2002;70:5759-5769). Although in all murine and non-human primate experiments conducted to date adverse reactions to the vaccine strains have never been observed, ClyA still possesses hemolytic activity in vitro, and could therefore theoretically be considered a virulence factor with the potential to cause adverse reactions in humans. To remove hemolytic activity from *S.* Typhi *clyA*, it was elected to molecularly evolve a codon-optimized allele of *clyA* genetically fused to the *gfpuv* allele of pSEC92*gfpuv*. Fusion of foreign protein domains to the carboxyl terminus of ClyA reduces the hemolytic activity of the ClyA domain, probably by perturbing the natural folding of ClyA and possibly affecting outer membrane vesicle formation. It is therefore believed that evolving ClyA in the context of a fusion domain would increase chances of successfully switching this domain with antigens from *C. difficile* to preserve export of these

vaccine antigens. Fusion of ClyA to the fluorescent reporter GFPuv was chosen since it is well documented that GFPuv will not fluoresce in the context of improperly folded upstream domains (Waldo GS, Standish BM, Berendzen J, Terwilliger TC. Rapid protein-folding assay using green fluorescent protein. *Nat Biotechnol.* 1999;17:691-695).

Subjecting *clyA* to a single round of molecular evolution yielded 111 clones that appeared non-hemolytic on sheep blood agar plates, but still fluoresced. Sequencing of these mutagenized constructs revealed that 18 recombinants had single amino acid substitutions that removed hemolytic activity. These 18 constructs expressing non-hemolytic ClyA (ClyA*) were retransformed into DH5α and export of ClyA*-GFPuv into the culture supernatant was examined using Western immunoblot analysis with anti-GFP polyclonal antibody. Six individual mutations (S195N, I198N, A199D, E204K, E204D, G205D) abrogated hemolytic activity while preserving export of ClyA*-GFPuv into the supernatant. Quantitative hemolytic assays performed on these 6 ClyA variants revealed that mutations S195N, I198N, and A199D each dramatically reduced hemolytic activity to 2-8% of wildtype (Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect Immun.* 1986;51:461-469). The G205D mutation reduced the hemolytic activity to less than 50%. Interestingly, the 2 mutations at residue 204 (E204K, E204D) reduced hemolytic activity to different degrees, depending on which amino acid replaced the wildtype glutamic acid. The E204D substitution reduced hemolytic activity to 30% of wildtype, while the E204K substitution reduced hemolysis to less than 2%. These results demonstrate that hemolytic activity and export activity can be separated, and illustrate the power of using random molecular evolution to engineer desired phenotypes.

A triple mutant *clyA** gene containing the mutations of I198N, A199D, and E204K was genetically engineered. Plasmids expressing single or triple mutant ClyA* were introduced into CVD 908-*htrA* and the resulting live vector constructs tested both for hemolytic activity and export of ClyA*-GFPuv into the culture supernatant. Export of the triple mutant fusion was identical to that observed for

wildtype ClyA-GFPuv, as judged by western immunoblot analysis of culture supernatants (Figure 3A); note that the absence of GroEL in the supernatants strongly suggests that ClyA fusions are being exported into the supernatant in the absence of detectable autolysis (Figure 3B).

The immunogenicity of these single and triple ClyA*-GFPuv mutants was then tested using the murine intranasal model of immunogenicity. Mice were primed with 5×10^9 CFU of plasmid-bearing live vectors on days 0 and 14, and boosted intramuscularly on day 42 with 0.5 μg purified GFPuv adsorbed to 0.5 mg Alhydrogel in a total volume of 50 μl. Results are shown in Figure 4. As expected, wildtype ClyA-GFPuv fusions encoded by pSEC91*gfpuv* were highly immunogenic, with serum IgG titers rising 100 fold after the second priming dose of live vector was given. The immunogenicity of the single I198N non-hemolytic mutant encoded by pI198N*gfpuv* was indistinguishable from that of the wildtype fusion. Surprisingly, the triple mutant ClyA*-GFPuv fusion encoded by pSEC93*gfpuv* was poorly immunogenic, eliciting GFPuv-specific titers 10 fold less than wildtype and single mutant constructs. These data suggest that if the folding of ClyA is significantly perturbed by the accumulation of point mutations that abolish hemolytic activity, the immunogenicity of the passenger domain (GFPuv in this case) is reduced.

Example 3. Immunogenicity in mice of PA83 delivered by SSB-stabilized plasmids in CVD 908-*htrA* and CVD 908.

To further improve the clinical acceptability of the live vector vaccine, a non-antibiotic plasmid selection system has been developed for use in attenuated bacterial vaccine strains. Typically, foreign proteins are expressed within live vectors from multicopy expression plasmids that do not encode transfer functions and are not considered to be self-transmissible. Antibiotic resistance markers are usually inserted into expression plasmids for selection purposes after introduction of plasmids into live vectors. Until recently, these resistance markers were considered to pose no risk for complicating or causing failure of clinical antimicrobial treatments for 3 important reasons: 1] the expression plasmids (and

accompanying resistance markers) could not be efficiently mobilized from live vector donors to a recipient, 2] the genes used encoded resistance to antibiotics not in widespread medical use, and 3] with no relevant antibiotic selective pressure, even rare plasmid transfers would not lead to *de novo* resistances becoming established within a new bacterial population (Levine, M. M., J. B. Kaper, H. Lockman, R. E. Black, M. L. Clements, and S. Falkow. 1983. Recombinant DNA risk assessment studies in humans: efficacy of poorly mobilizable plasmids in biologic containment. J. Infect. Dis. 148:699-709). However, the Food and Drug Administration is now recommending that all vaccines intended for use as mucosal vaccines in humans be free of resistance to antibiotics. Therefore, the clinical acceptability of the live vector vaccines has been improved by engineering an ssb--derived selection system for the expression plasmids.

A set of isogenic expression plasmids was engineered in which PA83 fused to ClyA was expressed from SSB-stabilized medium copy (pSEC91-83S, ~ 15 copies per chromosomal equivalent) or low copy (pSEC10-83S, ~ 5 copies per chromosomal equivalent) plasmids (Figure 5). Plasmids were introduced into either CVD 908-*htrAssb* or the less attenuated parent CVD 908*ssb*. As a control, the immunogenic CVD 908-*htrA* (pSEC91-83) was included from previous non-human primate studies. Immunogenicity of these constructs was tested in BALB/c mice primed intranasally on days 0 and 14 with 5 x 10^9 cfu of live vector and boosted intramuscularly 28 days later (day 42) with 10 μg purified PA83 (List Biochemical Laboratories) adsorbed to Alhydrogel in a total volume of 50 μl. Preliminary results with pooled sera are shown in Table 3 (serum IgG) (see Galen JE, M. Chinchilla, M.F. Pasetti, J.Y. Wang, L. Zhao, I. Arciniega-Martinez, D.J. Silverman, and M.M. Levine. Mucosal immunization with attenuated *Salmonella* Typhi expressing anthrax PA83 primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine. *J. Infect. Dis.* 2009. 199: 326-35).

ıti-PA83 IgG responses in pooled serum from mice immunized intranasally with ssing *S.* Typhi live vectors and boosted with purified PA83.

ın	Plasmid Copy number	Foreign Antigen	SSB?	Day 41 (pre-boost)	Day 49 (1 wk post boost)	Day 55 (2 wks post boost)	Day 70 (4 wks post boost)
ssb(pSEC91dS)	~15	(-) control	YES	25	1,105	5,690	41,824
ssb(pSEC91-83S)	~15	ClyA-PA83	YES	25	2,944	80,710	598,700
ssb(pSEC10-83S)	~5	ClyA-PA83	YES	4,436	314,166	475,045	209,389
sb(pSEC91dS)	~15	(-) control	YES	25	25	8,484	206,628
sb(pSEC91-83S)	~15	ClyA-PA83	YES	2,631	296,222	630,795	1,180,372
sb(pSEC10-83S)	~5	ClyA-PA83	YES	18,431	856,485	1,219,323	504,111
pSEC91-83)	~15	ClyA-PA83	NO	442.9	108,283	108,283	755,215

Surprisingly, humoral responses engendered using CVD 908-*htrAssb*(pSEC91-83S), carrying an SSB-stabilized medium copy expression plasmid were clearly lower than responses elicited by the conventional CVD 908-*htrA*(pSEC91-83) live vector, carrying the kanamycin resistance medium copy pSEC91-83 plasmid (group 2 versus group 7). Prior to boosting (day 41), anti-PA83 responses were 10 fold lower for SSB-stabilized constructs versus conventional constructs, and responses for these two strains did not converge until 4 weeks after the boost. However, when the copy number for SSB-stabilized plasmids was reduced, serum responses with CVD 908-*htrAssb*(pSEC10-83S) rose 10 fold versus conventional CVD 908-*htrA*(pSEC91-83) on day 41, and remained elevated 2 weeks after boosting (group 3 versus group 7). These data clearly suggest that as copy number is reduced, the immunogenicity of the foreign antigen improves. It is believed that this effect is due to the reduced metabolic burden associated with the lower copy number of pSEC10-83S. Indeed, when pSEC10-83S is carried by the less attenuated CVD 908*ssb* live vector, immunogenicity of the foreign antigen increases yet again and remains elevated 4 weeks post boost (group 3 versus group 6). Here it is stressed that CVD 908-*htrAssb* and CVD 908*ssb* are isogenic strains, differing only in the deletion of a single *htrA* chromosomal locus. However, identical expression plasmids elicit higher serum antibody responses in the less attenuated CVD 908*ssb* live vector than when carried by CVD 908-*htrAssb*. It is believedthat any metabolic burden still

associated with pSEC10-83S is compensated for in the less attenuated background of CVD 908*ssb*. Based on these highly encouraging data, the immunogenicity will be examined of *C. difficile* antigens expressed using low copy number pSEC10 expression plasmids, genetically stabilized with the SSB system, and carried either by CVD 908-*htrAssb* or CVD 908*ssb*.

Example 4. Genetic stability of SSB-stabilized plasmids expressing GFPuv.

The 10-fold drop in immunogenicity of ClyA-PA83 delivered using SSB-stabilized pSEC91-83S versus conventional pSEC91-83 suggested that perhaps SSB-stabilized higher copy number plasmids encoding a potentially toxic foreign protein may lead to genetic instability and perhaps re-arrangements or deletions of foreign genes from the expression plasmid. To investigate this further, previously described GFPuv-encoding plasmids pGEN222 and pGEN206 were tested (Galen, J. E., J. Nair, J. Y. Wang, S. S. Wasserman, M. K. Tanner, M. Sztein, and M. M. Levine. 1999. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella* Typhi CVD 908-*htrA*. *Infect. Immun.* 67:6424-6433; Stokes, M. G., R. W. Titball, B. N. Neeson, J. E. Galen, N. J. Walker, A. J. Stagg, D. C. Jenner, J. E. Thwaite, J. P. Nataro, L. W. Baillie, and H. S. Atkins. 2007. Oral administration of a *Salmonella* enterica-based vaccine expressing *Bacillus anthracis* protective antigen confers protection against aerosolized *B. anthracis*. *Infect. Immun.* 75:1827-1834). The expected copy number of pGEN222 is 15 copies per chromosomal equivalent, and that of pGEN206 is 5 copies per chromosomal equivalent. Isogenic SSB-stabilized versions of pGEN222 and pGEN206 were engineered, as shown in Figure 6A. After electroporating these four isogenic expression plasmids into CVD 908-*htrA*, the desired live vector strains were recovered, and an in vitro plasmid stability experiment was carried out. Frozen stocks were streaked onto appropriately supplemented solid media, and incubated at 30°C for 48 hours to obtain isolated colonies. 2-3 fluorescing colonies were then inoculated into 20 ml liquid medium without selection and incubated shaking at 225 rpm overnight at 30°C (0 hour starting cultures for serial passages). Overnight starter cultures were then diluted 1:50 into fresh non-selective medium, incubated for 24 hr at 37°C, and then serially passaged every 24

hours in the same way for 5 days. Viable counts were recovered on non-selective medium, and the percentage of fluorescing colonies determined, as reported in Figure 6B. As expected, conventional plasmids were unstable, with less than 20% of strains carrying pGEN222 remaining fluorescent after passage for 24 hr; live vectors maintained the lower copy number pGEN206 for 48 hr, but then plasmid-bearing strains dropped precipitously on day 3. Surprisingly, the fluorescence of strains carrying SSB-stabilized pGEN222S dropped precipitously after 24 hr, despite the fact that these plasmids encode the essential *ssb* gene. It is expected that the higher copy number pGEN222S constructs encoding GFPuv are toxic, possibly leading to non-homologous integration into the chromosome accompanied by loss of the foreign antigen-encoding gene (in this case *gfpuv*). Notably, the observed stability of the low copy number SSB-stabilized pGEN206S plasmid was excellent after 120 hours of culturing in the absence of selection. This observation potentially explains the superior immunogenicity of pSEC10-83S constructs versus the higher copy number pSEC91-83S plasmids reported in Table 2, and again supports the use of pSEC10 plasmids for expression of potentially toxic *C. difficile* foreign antigens.

Example 5. Preparation of an attenuated bacterial strain derived from *S.* Typhi Ty2 and carrying deletions in both *guaBA* and *htrA*.

A new vaccine strain will be constructed, carrying deletions in *guaBA* and *htrA*, which will be attenuated within half the logarithmic value of CVD 908-*htrA*.

Deletion cassettes, targeting *guaBA* and *htrA*, for use with the lambda Red-mediated site-directed mutagenesis method will be used to successfully delete either *guaBA* or *htrA* from wildtype *S.* Typhi Ty2 (Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A.* 2000;97:6640-6645). The deletion cassettes will be introduced into a single strain, creating CVD 910. The degree of attenuation of CVD 910 will be assessed by comparing the minimum lethal dose causing death in 50% of a group of BALB/c mice (LD50) for CVD 910 versus both CVD 908-*htrA* and its wildtype parent Ty2, using the hog gastric mucin intraperitoneal murine challenge model. For this model, the guidelines

recommended in the Code of Federal Regulations for Food and Drugs, Title 21, Part 620.13 (*c-d*), 1986 for intraperitoneal challenge of mice with *S.* Typhi will be followed. Using this method the LD50 of an attenuated strain of Ty2, carrying a single deletion in *guaBA*, was observed to be 7.7 x 10^7 CFU, versus an LD50 of 140 CFU for wildtype Ty2 and >5 x 10^9 CFU for CVD 908-*htrA* (unpublished observations) (Wang JY, Pasetti MF, Noriega F et al. Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated D*guaBA Salmonella enterica* serovar Typhi strain CVD 915. *Infect Immun.* 2001;69:4734-4741). An LD50 of between 1 and 5 x 10^9 CFU will be acceptable for use of CVD 910 in the development of the proposed *C. difficile* vaccine.

The immunogenicity of CVD 910 will then be compared to that of CVD 908-*htrA* using the murine intranasal model of immunogenicity. Adult BALB/c mice will be randomized into three groups and immunized with either CVD 910, CVD 908-*htrA*, or PBS. Mice will be immunized intranasally on days 0 and 14 with 5 x 10^9 CFU of attenuated vaccine. Serum IgG antibody responses to both lipopolysacchriade (LPS) and flagella (H antigen) will be measured on days 0, 14, 28, and 42 to confirm comparable humoral immune responses against CVD 910 versus CVD 908-*htrA*.

One antigen (e.g., TcdB) will be expressed on a low copy number SSB-stabilized expression plasmid, and the other antigens (TcdA and CdtB) will be integrated into the deleted loci of the attenuated *S.* Typhi strain derived from wildtype Ty2

Example 6. Expression of cell-binding domains from *C. difficile* enterotoxins A (TcdA), B (TcdB), or binary toxin (CdtB) from plasmids in an attenuated *S.* Typhi vaccine strain and testing for toxin-specific antibody responses in mice using a heterologous prime-boost strategy.

SSB-stabilized low copy (~5 copies per chromosomal equivalent) expression plasmids encoding either a truncated version of the Cell Binding Domain of TcdA (14CBD/A) or the full-length Cell Binding Domain of TcdB (CBD/B) were constructed. Plasmids were constructed encoding 14CBD/A and

CBD/B expressed either cytoplasmically or exported out of the cytoplasm as a fusion to ClyA. Transcription of these cassettes is osmotically controlled by a P_{ompC} promoter (Galen JE, Nair J, Wang JY et al. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella* Typhi CVD 908-*htrA*. *Infect Immun.* 1999;67:6424-6433). When introduced into the live vector CVD 908-*htrAssb*, excellent expression as judged by western immunoblotting using toxin specific sera was observed (see Example 1), and CVD 908-*htrAssb* expressing ClyA-CBD/B fusions proved immunogenic using a murine intranasal model of immunogenicity (Table 1). Since ClyA possesses hemolytic activity in vitro, ClyA can theoretically be considered a virulence factor, a concern that could complicate moving forward into future clinical trials. To improve the clinical acceptability of the trivalent vaccine strain, the immunogenicity of *C. difficile* antigens fused to a modified version of ClyA, in which hemolytic activity has been removed by introducing the substitution of a hydrophilic arginine residue for the hydrophobic wildtype isoleucine residue 198 (I198N) will be examined; preliminary data indicate that this substitution abolishes hemolytic activity while preserving export of a carboxyl-terminal biologically active fusion of GFPuv. This non-hemolytic allele is referred to as ClyA*, encoded by *clyA**. It will be confirmed that all ClyA* fusions are non-hemolytic using a previously described quantitative hemolysis assay (Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect Immun.* 1986;51:461-469).

Six low copy SSB-stabilized expression plasmids encoding the cell-binding domains of either TcdA (14CBD/A), TcdB (CBD/B), or binary toxin (CBD/BT), and expressed either cytoplasmically or exported from the cytoplasm as fusions to non-hemolytic ClyA (I198N) will be constructed. All cassettes will be transcriptionally controlled by the osmotically inducible P_{ompC} promoter (Galen JE, Nair J, Wang JY et al. Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella* Typhi CVD 908-*htrA*. *Infect Immun.* 1999;67:6424-6433). *ssb* from CVD 910 will be deleted to generate a live vector analogous to previous CVD 908-*htrAssb*, creating CVD 910*ssb*. Upon completion,

the six expression plasmids will be introduced into CVD 910*ssb* by electroporation as previously described (Galen JE, Wang JY, Chinchilla M et al. A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in *Salmonella enterica* serovar Typhi live vectors. *Infect Immun.* 2010;78:337-347). The immunogenicity of the resulting strains will be tested using a heterologous mucosal prime-parenteral boost immunization strategy to identify the most immunogenic way to deliver each of the *C. difficile* antigens (i.e. fused or unfused). Adult BALB/c mice will be randomized into 7 groups and primed with CVD 910*ssb* live vectors expressing the following *C. difficile* antigens:

group 1) ClyA*-14CBD/A fusion protein,
group 2) Unfused 14CBD/A,
group 3) ClyA*-CBD/B fusion protein,
group 4) Unfused CBD/B,
group 5) ClyA*-CBD/BT fusion protein
group 6) Unfused CBD/BT
group 7) CVD 910*ssb* carrying an empty plasmid encoding SSB

Mice will be primed intranasally on days 0 and 14 with a dose of 5×10^9 CFU, and boosted intramuscularly on day 42 with homologous antigen adsorbed to alum; use of this immunization strategy was demonstrated by our group to elicit neutralizing antibody responses against anthrax toxin when used with both mice and non-human primates (Galen JE, Chinchilla M, Pasetti MF et al. Mucosal immunization with attenuated *Salmonella enterica* serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine. *J Infect Dis.* 2009;199:326-335). Boosting antigens will be purified. Serum IgG antibody responses will be measured by ELISA on days 0, 14, 28, 42, 56, and 70. To more fully characterize the breadth of immune responses, enterotoxin-specific fecal IgA antibody responses will be determined on day 70, as an indicator of mucosal immunity (Lasaro MO, Luiz WB, Sbrogio-Almeida ME, Ferreira LC. Prime-boost vaccine regimen confers protective immunity to human-derived enterotoxigenic *Escherichia coli. Vaccine.* 2005;23:2430-2438). However, a primary endpoint for immunity against TcdA and TcdB will be serum neutralizing antitoxin responses

on days 56 and 70, measured using IMR-90 cells which are sensitive to both toxin A and toxin B. Biologically active holotoxins A and B, to be used in the neutralization assays and as the coating antigen for ELISA analyses, will be provided. Neutralization assays have been established for other toxins including tetanus toxin and anthrax toxin (Galen JE, Gomez-Duarte OG, Losonsky G et al. A murine model of intranasal immunization to assess the immunogenicity of attenuated *Salmonella* Typhi live vector vaccines in stimulating serum antibody responses to expressed foreign antigens. *Vaccine.* 1997;15:700-708; Galen JE, Chinchilla M, Pasetti MF et al. Mucosal immunization with attenuated *Salmonella enterica* serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine. *J Infect Dis.* 2009;199:326-335). Anti-Cdt immunity will be examined by measuring serum IgG antibody responses against CBD/BT on days 56 and 70 for fused versus unfused CBD/BT, as well as Cdt-specific fecal IgA on day 70 (Lasaro MO, Luiz WB, Sbrogio-Almeida ME, Ferreira LC. Prime-boost vaccine regimen confers protective immunity to human-derived enterotoxigenic *Escherichia coli*. *Vaccine.* 2005;23:2430-2438).

If expression of a truncated version of the carboxyl terminus of toxin A does not elicit neutralizing antibodies, then additional cassettes will be inserted encoding the remaining repeat regions to improve folding and immunogenicity of the larger protein. In the unlikely event that the I198N substitution diminishes the immunogenicity of *ClyA**-CBD/B, possibly indicating improper folding of this fusion compared to previous success with hemolytic ClyA-CBD/B, other previously identified non-hemolytic mutations including A199D or E204K will be tested.

Example 7. Chromosomal integration of cassettes encoding *C. difficile* antigens generates a multivalent strain and testing the strain in mice using a heterologous prime-boost strategy.

Immunogenic cassettes will be integrated into either the Δ*guaBA* or Δ*htrA* locus of CVD 910*ssb*, to compare the immunogenicity of chromosomal integrations versus antigen-specific immunogenicity elicited by plasmid-based

expression. For the engineering of the Δ*guaBA* and Δ*htrA* attenuating deletions of CVD 910*ssb*, only the open reading frames were deleted, leaving the original promoters for these sites intact. Therefore, to compensate for loss of copy number with chromosomal expression, cassettes will include the P_{ompC} promoter from the low copy expression plasmids, such that integration into Δ*guaBA* or Δ*htrA* results in nested promoters controlling inducible expression of a given cassette at two levels. For example, integration of a P_{ompC}-*clyA*-14cbd/a* cassette (encoding the ClyA*-14CBD/A fusion) into Δ*guaBA* of CVD 910*ssb* would result in inducible transcription of *clyA*-14cbd/a* with both growth rate and osmolarity, as shown in FIG. 9. This will generate a bivalent live vector to be used in Example 8 for creation of the final trivalent live vector vaccine.

All chromosomal integrations will first be introduced into CVD 910, prior to final introduction of the *ssb* deletion to generate strains that can be compared to plasmid-bearing CVD 910*ssb* strains. Three separate mouse experiments will then be carried out, testing the immunogenicity of chromosomally integrated genetic cassettes; the objective of these experiments is to identify which chromosomal integrations elicit antigen-specific immunogenicity comparable to that observed for plasmid-based expression. It is assumed that the most immunogenic cassettes encode fusions of ClyA* fused to 14CBD/A (encoded by *clyA*-14cbd/a*) or CBD/B (encoded by *clyA*-cbd/b*), while CBD/BT remains unfused. For all experiments, BALB/c mice will be randomly assorted into groups, immunized intranasally on days 0 and 14 with 5 x 10^9 CFU of live vector, and then boosted intramuscularly on day 42 with homologous purified antigen adsorbed to alum. Study 1, below, will determine how chromosomal expression of optimized genetic cassettes encoding *C. difficile* antigens integrated into the *guaBA* locus compares with plasmid-based expression of the identical antigen cassette:

group 1) 910*ssb* Δ*guaBA*::*clyA*-14cbd/a*

group 2) 910*ssb* (pClyA*-14CBD/A)

group 3) 910*ssb* Δ*guaBA*::*clyA*-cbd/b*

group 4) 910*ssb* (pClyA*-CBD/B)

group 5) 910*ssb* Δ*guaBA*::*cbd/bt*

group 6) 910*ssb* (pCBD/BT)

group 7) 910*ssb* carrying only the empty plasmid encoding SSB

Study 2 examines how chromosomal expression of *C. difficile* antigens integrated into the *htrA* locus will compare with plasmid-based expression of the identical antigen cassette:

group 1) 910*ssb* Δ*htrA*::*clyA*-14cbd/a*

group 2) 910*ssb* (pClyA*-14CBD/A)

group 3) 910*ssb* Δ*htrA*::*clyA*-cbd/b*

group 4) 910*ssb* (pClyA*-CBD/B)

group 5) 910*ssb* Δ*htrA*::*cbd/bt*

group 6) 910*ssb* (pCBD/BT)

group 7) 910*ssb* carrying only the empty plasmid encoding SSB.

For Study 3, the most immunogenic chromosomal integrations identified in the previous two previous experiments will be introduced into a single bivalent strain and compare the immunogenicity of both integrated antigens to plasmid-based expression of the identical antigen cassette in monovalent strains. It is assumed that Δ*guaBA*::*clyA*-14cbd/a* and Δ*htrA*::*clyA*-cbd/b* elicit antigen-specific immunogenicity comparable to that observed for plasmid-based expression. Therefore, in this particular scenario, it is assumed that the immunogenicity of chromosomally integrated CBD/BT will be found to be significantly less than plasmid-based expression, so pCBD/BT will be used to construct the final trivalent vaccine. Therefore, BALB/c mice will be randomly assorted into 4 groups for intranasal immunization with:

group 1) 910*ssb* Δ*guaBA*::*clyA*-14cbd/a* Δ*htrA*::*clyA*-cbd/b*

group 2) 910*ssb* (pClyA*-14CBD/A)

group 3) 910*ssb* (pClyA*-CBD/B)

group 4) 910*ssb* carrying only the empty plasmid encoding SSB

Mice will be immunized intranasally with live vectors on days 0 and 14 and boosted intramuscularly with a mixture of the three purified antigens adsorbed to alum. For all 3 studies, toxin-specific serum IgG antibody responses will be measured on days 0, 14, 28, 42, 56 and 70, and fecal IgA on day 70. The primary endpoints will be 1] serum neutralizing antitoxin responses against TcdA and TcdB

on days 56 and 70, measured using IMR-90 cells, and 2] Cdt-specific fecal IgA on day 70.

These studies will allow final construction of the multivalent vaccine, in which two *C. difficile* antigens are efficiently expressed from the chromosome and the remaining antigen is expressed from a low copy SSB-stabilized plasmid. It is possible that chromosomal integration of *C. difficile* antigens fused to ClyA* will integrate into the wildtype *clyA* locus present in CVD 910*ssb*, rather than *guaBA* or *htrA*. If the presence the wildtype *clyA* allele causes improper integration of incoming cassettes, wildtype *clyA* from the live vector chromosome can be deleted. This deletion is not expected to further attenuate the resulting live vector.

Example 8. Immunization of hamsters with a trivalent live vector vaccine expressing cell-binding domains from TcdA, TcdB, and Cdt, followed by oral challenge with *C. difficile* spores.

An SSB-stabilized expression plasmid encoding the remaining *C. difficile* antigen will be introduced into the final bivalent live vector vaccine identified in the above example. Assuming the bivalent strain to be 910*ssb* Δ*guaBA*::*clyA**-*14cbd/a* Δ*htrA*::*clyA***-cbd/b*, a trivalent vaccine is represented in Figure 10. Prior to challenge experiments, the immunogenicity of the trivalent live vector in Syrian golden hamsters immunized intranasally will be established. Two experiments will be performed, testing both a heterologous mucosal live vector prime-intramuscular boost immunization strategy (as was done for mice; Study 1), as well as reversing the order and administering an intramuscular prime-mucosal live vector boost (Study 2). Using live vectors as a mucosal boost tests an immunization strategy that more closely resembles the proposed manner in which the trivalent vaccine would be used in a clinical setting to vaccinate patients recovering from a primary CDI.

Study 1:

group 1) mucosal priming with trivalent 910*ssb* expressing 14CBD/A, CBD/B, and CBD/BT antigens, and boosting with purified antigens

group 2) mucosal priming with bivalent 910*ssb* expressing 14CBD/A and CBD/B antigens only, and boosting with purified antigens

group 3) mucosal priming with monovalent 910*ssb* expressing only CBD/BT and boosting with purified antigens

group 4) mucosal priming with 910*ssb* carrying only the empty plasmid encoding SSB, boosting with purified antigens

Study 2:

group 1) priming with purified antigens, mucosal boosting with trivalent 910*ssb* expressing 14CBD/A, CBD/B, and CBD/BT

group 2) priming with purified antigens, mucosal boosting with bivalent 910*ssb* expressing 14CBD/A and CBD/B antigens only

group 3) priming with purified antigens, mucosal boosting with monovalent 910*ssb* expressing only CBD/BT

group 4) priming with purified antigens, mucosal boosting with 910*ssb* carrying only the empty plasmid encoding SSB

For priming with live vectors (Study 1), animals will be immunized intranasally on days 0 and 14 with $5x10^9$ CFU of the vaccine strains and boosted intramuscularly with purified antigens plus alum on day 42. For boosting with live vectors (Study 2), animals will be primed intramuscularly on day 0 with purified antigens plus alum and boosted on days 28 and 42 with live vectors. As with the mouse experiments, toxin-specific IgG antibody responses will be measured by ELISA on days 0, 14, 28, 42, 56, and 70, with serum neutralizing enterotoxin responses measured on days 56 and 70.

Upon confirming the most immunogenic of these two strategies in hamsters, an identical intranasal immunization protocol will be carried out, prior to orogastric challenge with *C. difficile* spores. Two weeks after administration of the booster dose (day 56), hamsters will be then treated with clindamycin administered intraperitoneally 24 hrs prior to orogastric challenge with 100 colony-forming units of *C. difficile* spores. Animals will be scored for mortality over a 5 day period. With this protocol, 100% of unimmunized animals succumb to disease between 36 and 72 hrs after spore challenge (Babcock GJ, Broering TJ, Hernandez HJ et al. Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. *Infect Immun.* 2006;74:6339-6347). It is expected that there will be 100% mortality for animals in group 4 that receive only

a single dose of purified antigens. It is expected that hamsters in group 1, primed with trivalent live vectors and boosted with purified antigens, will be fully protected from challenge with an epidemic strain of *C. difficile*. By contrasting protection in group 1 with that of groups 2 and 3, the relative contributions to protection of enterotoxin immunity (group 2) versus immunity to binary toxin (group 3) will be determined.

Studies 1 and 2 of this example can be repeated using C57BL/6 mice and the murine *C. difficile* challenge model recently described by Chen et al. (Chen X, Katchar K, Goldsmith JD et al. A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology*. 2008;135:1984-1992).

It is expected that these examples will result in an *S.* Typhi-based live vector vaccine against CDI and it is further expected to provide broad humoral and mucosal immunogenicity in humans that may not be easily achieved using purified protein subunit vaccines.

While the invention has been described with reference to certain particular examples and embodiments herein, those skilled in the art will appreciate that various examples and embodiments can be combined for the purpose of complying with all relevant patent laws (e.g., methods described in specific examples can be used to describe particular aspects of the invention and its operation even though such are not explicitly set forth in reference thereto).

WHAT IS CLAIMED IS:

1. A method of inducing an immune response in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *Salmonella* Typhi vector comprising a cell binding domain of TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof and a cell binding domain of TcdB toxin (CBD/B) of *Clostridium difficile* or an antigenic fragment thereof.

2. The method of claim 1, wherein the *S.* Typhi vector further comprises the cell-binding subunit (CdtB) of the *Clostridium difficile* colonization factor binary toxin, or an antigenic fragment thereof.

3. The method of claim 2, wherein the TcdB toxin (CBD/B) is expressed on a plasmid in *S.* Typhi.

4. The method of claim 3, wherein the plasmid has a non-antibiotic based plasmid selection system.

5. The method of claim 4, wherein the plasmid expresses a gene that is essential for the growth of *S.* Typhi and has been chromosomally mutated in *S.* Typhi.

6. The method of claim 5, wherein the gene encodes single stranded binding protein (SSB).

7. The method of claim 6, wherein the TcdA toxin (CBD/A) and CdtB from binary toxin are chromosomally integrated in *S.* Typhi.

8. The method of claim 7, wherein the TcdA toxin (CBD/A), the TcdB toxin (CBD/B), and CdtB from binary toxin are fused to cytolysin A (ClyA) protein from *S.* Typhi to facilitate their export from the cell.

9. The method of claim 8, wherein ClyA is mutated to reduce hemolytic activity of ClyA.

10. The method of claim 9, wherein the ClyA mutant is selected from the group consisting of ClyA I198N and ClyA C285W.

11. The method of claim 8, wherein the TcdA toxin (CBD/A) and CdtB from binary toxin are inserted into the *guaBA* locus of *S.* Typhi.

12. The method of claim 8, wherein CdtB from binary toxin is inserted into the *htrA* locus of *S.* Typhi and the TcdA toxin (CBD/A) is inserted into the *guaBA* locus of *S.* Typhi.

13. A *Clostridium difficile* vaccine comprising a live *S.* Typhi vector comprising a cell binding domain of TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof and a cell binding domain of TcdB toxin (CBD/B) of *Clostridium difficile* or an antigenic fragment thereof.

14. The vaccine of claim 13, wherein the *S.* Typhi vector further comprises the cell-binding subunit (CdtB) of the *Clostridium difficile* colonization factor binary toxin, or an antigenic fragment thereof.

15. The vaccine of claim 14, wherein the TcdB toxin (CBD/B) is expressed on a plasmid in *S.* Typhi.

16. The vaccine of claim 15, wherein the plasmid has a non-antibiotic based plasmid selection system.

17. The vaccine of claim 16, wherein the plasmid expresses a gene that is essential for the growth of *S.* Typhi and has been chromosomally mutated in *S.* Typhi.

18. The vaccine of claim 17, wherein the gene encodes single stranded binding protein (SSB).

19. The vaccine of claim 18, wherein the TcdA toxin (CBD/A) and CdtB from binary toxin are chromosomally integrated in *S.* Typhi.

20. The vaccine of claim 19, wherein the TcdA toxin (CBD/A), the TcdB toxin (CBD/B) and CdtB from binary toxin are fused to cytolysin A (ClyA) protein from *S.* Typhi to facilitate export from the cell.

21. The vaccine of claim 20, wherein ClyA has been mutated to reduce hemolytic activity of ClyA.

22. The vaccine of claim 21, wherein the ClyA mutant is selected from the group consisting of ClyA I198N and ClyA C285W.

23. The vaccine of claim 19, wherein the TcdA toxin (CBD/A) and CdtB from binary toxin are inserted into the *guaBA* locus of *S.* Typhi.

24. The vaccine of claim 19, wherein CdtB from binary toxin is inserted into the *htrA* locus of *S.* Typhi and the TcdA toxin (CBD/A) is inserted into the *guaBA* locus of *S.* Typhi.

25. The vaccine of claim 19, wherein one or more nucleic acid sequences encoding the TcdA toxin (CBD/A), the TcdB toxin (CBD/B) or CdtB from binary toxin are codon optimized for expression in *S.* Typhi.

26. A method of inducing an immune response against *Clostridium difficile* in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *S.* Typhi vector comprising a cell binding domain of TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof, a cell binding domain of TcdB toxin (CBD/B) of *Clostridium difficile* or an antigenic fragment thereof, and optionally further comprising CdtB from binary toxin of *Clostridium difficile* or an antigenic fragment thereof as a prime, and subsequently administering a boost comprising a polypeptide selected from the group consisting of TcdA toxin (CBD/A) or an antigenic fragment thereof, TcdB toxin (CBD/B) or an antigenic fragment thereof, and CdtB or an antigenic fragment thereof, and combinations thereof.

27. A method of preventing recurrence of a *Clostridium difficile* infection in a subject in need thereof, comprising administering to the subject an immunologically-effective amount of a live *S.* Typhi vector comprising a cell binding domain of TcdA toxin (CBD/A) of *Clostridium difficile* or an antigenic fragment thereof, a cell binding domain of TcdB toxin (CBD/B) of *Clostridium difficile* or an antigenic fragment thereof and optionally further comprising CdtB from binary toxin of *Clostridium difficile* or an antigenic fragment thereof.

28. The method of any one of claims 1, 26 or 27, wherein the *S.* Typhi live vector is administered mucosally.

Fig. 1

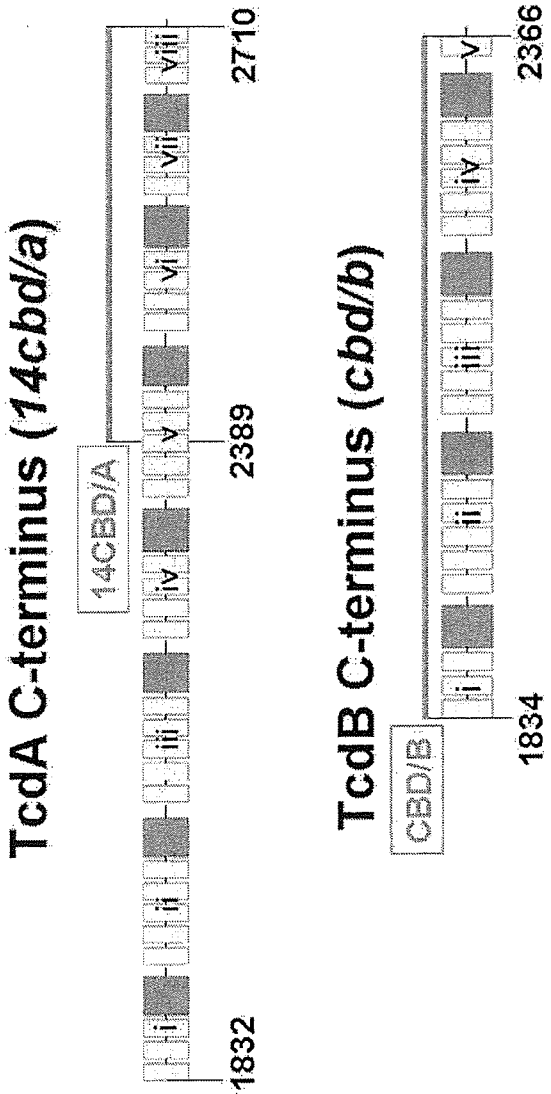
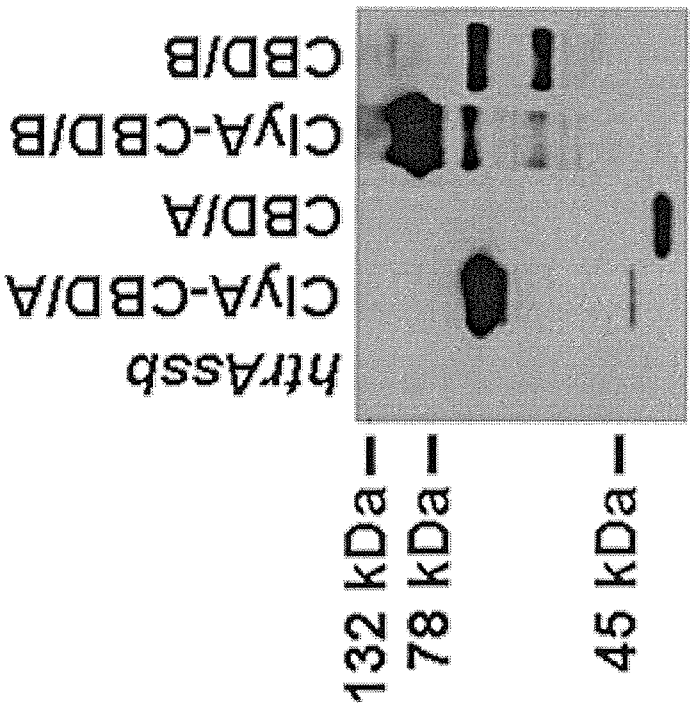

TcdA C-terminus (14cbd/a)
14CBD/A
i
ii
iii
iv
v
vi
vii
viii
1832
2389
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TcdB C-terminus (cbd/b)
CBD/B
i
ii
iii
iv
v
1834
2366

Fig. 2



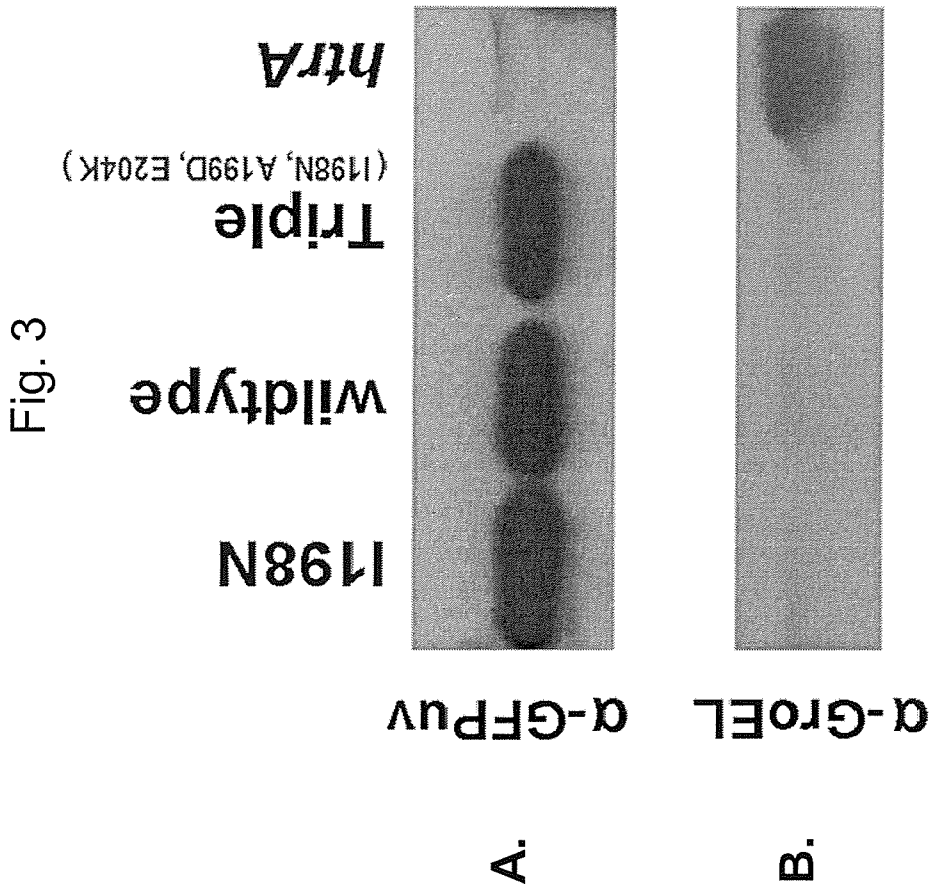

A.
B.
α-GFPuv
α-GroEL
I198N
wildtype
Triple
(I198N, A199D, E204K)
htrA

Fig. 3

Fig. 4

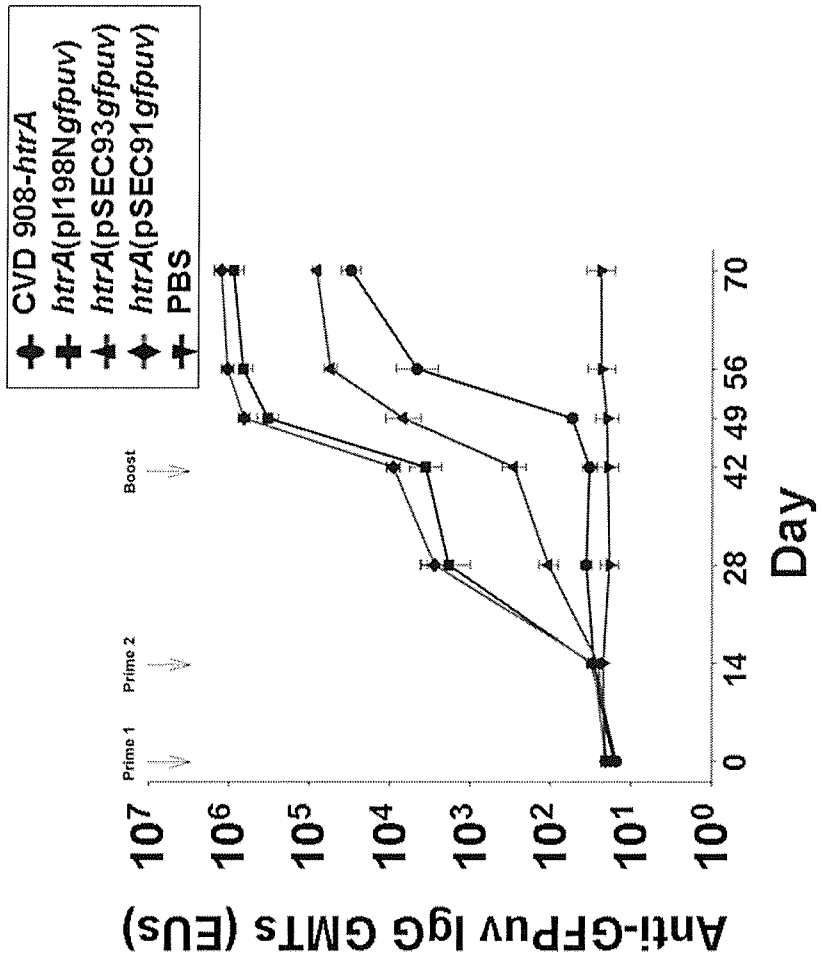

CVD 908-htrA
htrA(pI198Ngfpuv)
htrA(pSEC93gfpuv)
htrA(pSEC91gfpuv)
PBS
Prime 1
Prime 2
Boost
Anti-GFPuv IgG GMTs (EUs)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
14
28
42
49
56
70
Day

Fig. 5

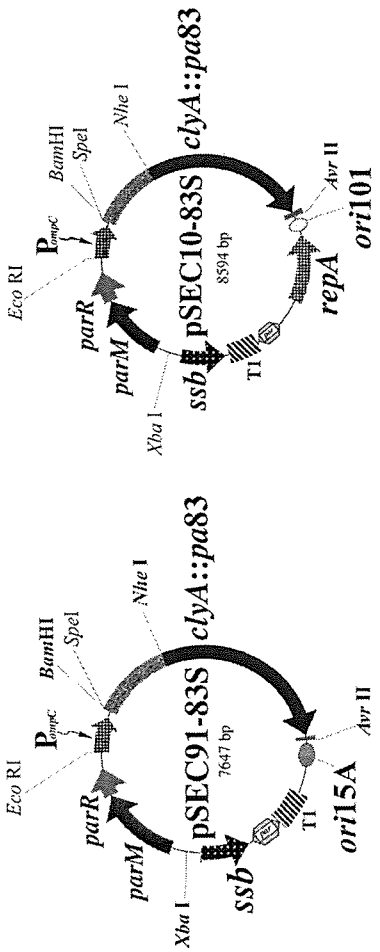

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Nhe I
BamHI
SpeI
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Fig. 6

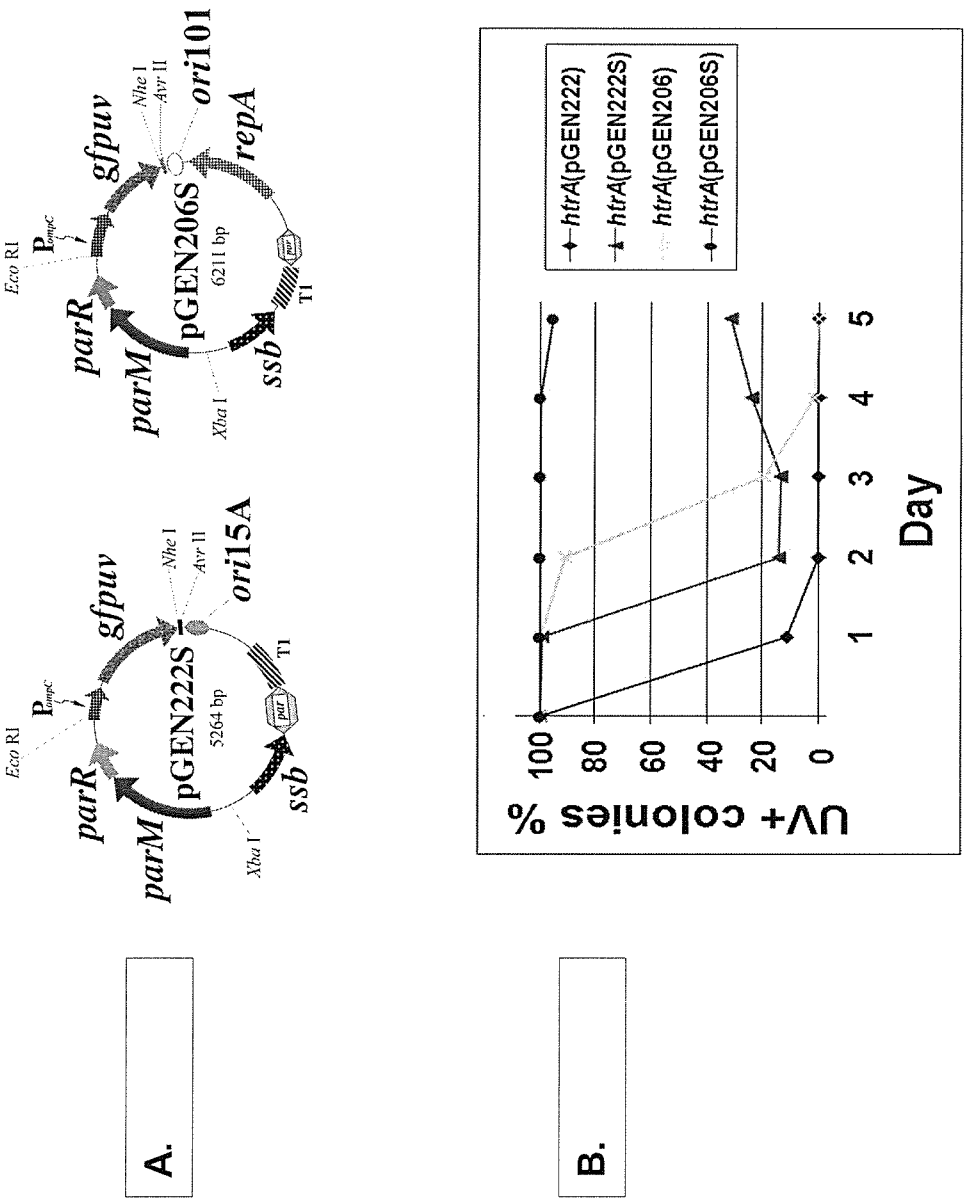

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repA
ori15A
ori101
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Eco RI
Xba I
Nhe I
Avr II
B.
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htrA(pGEN222)
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htrA(pGEN206)
htrA(pGEN206S)

Fig. 7

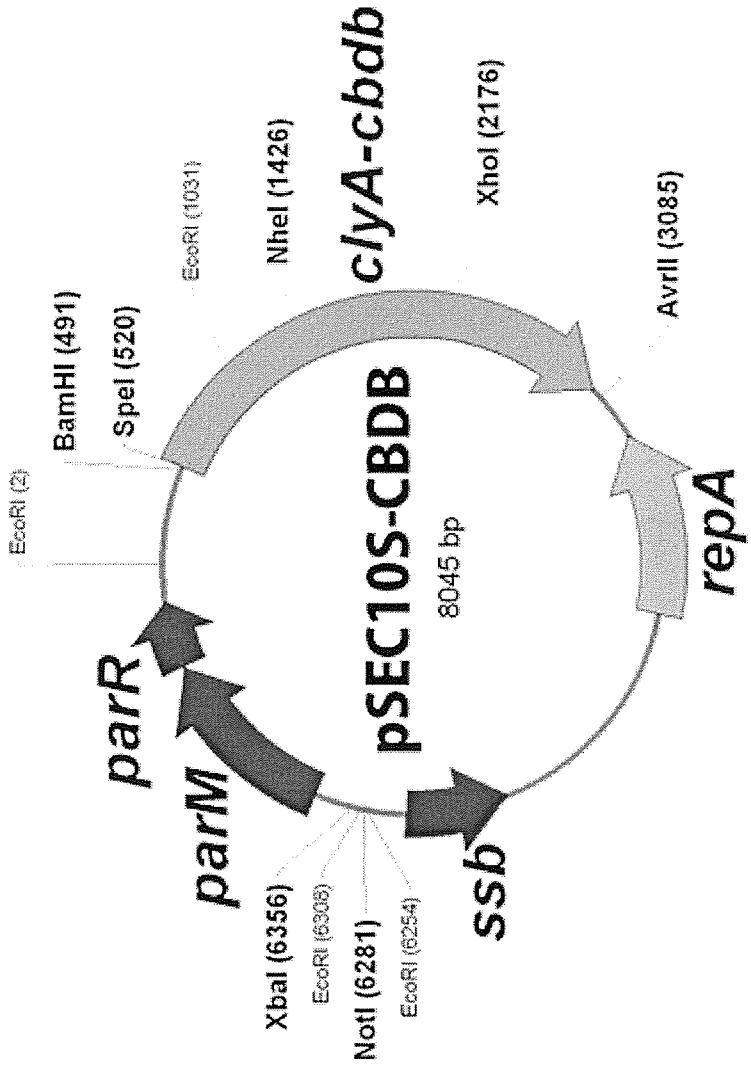

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clyA-cbdb
repA
ssb
parM
parR
EcoRI (2)
BamHI (491)
SpeI (520)
EcoRI (1031)
NheI (1426)
XhoI (2176)
AvrII (3085)
XbaI (6356)
EcoRI (6308)
NotI (6281)
EcoRI (6254)

Fig. 8

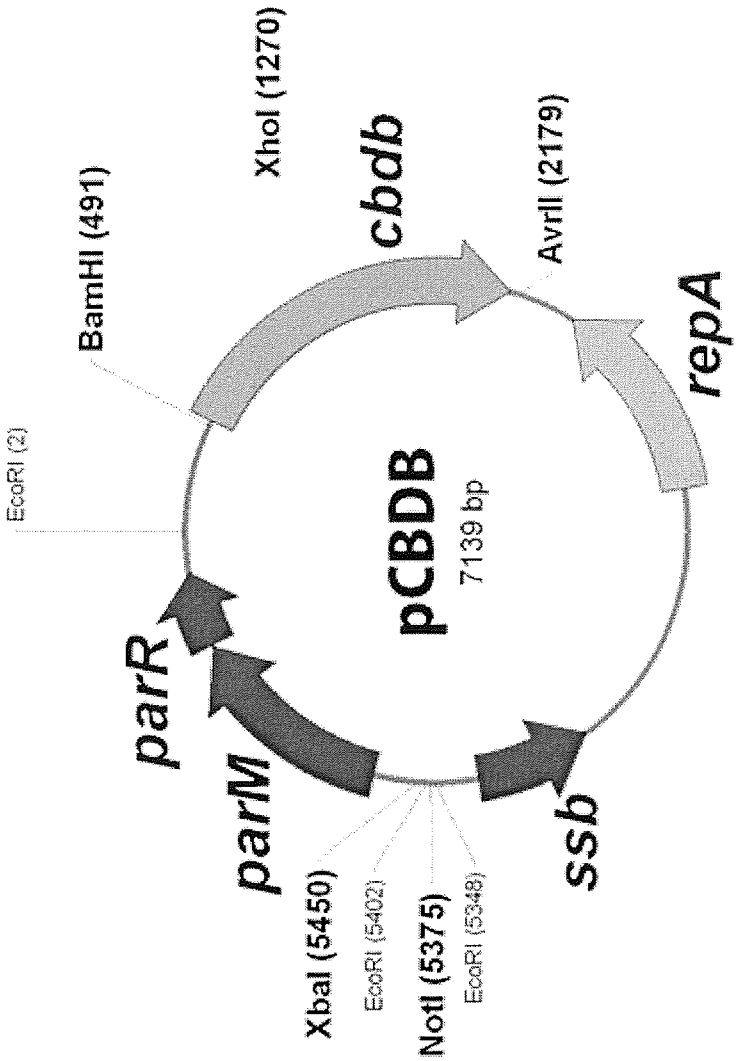

pCBDB
7139 bp
cbdb
repA
ssb
parM
parR
BamHI (491)
XhoI (1270)
AvrII (2179)
EcoRI (2)
XbaI (5450)
EcoRI (5402)
NotI (5375)
EcoRI (5348)

Fig. 9

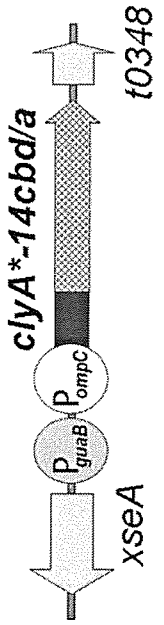
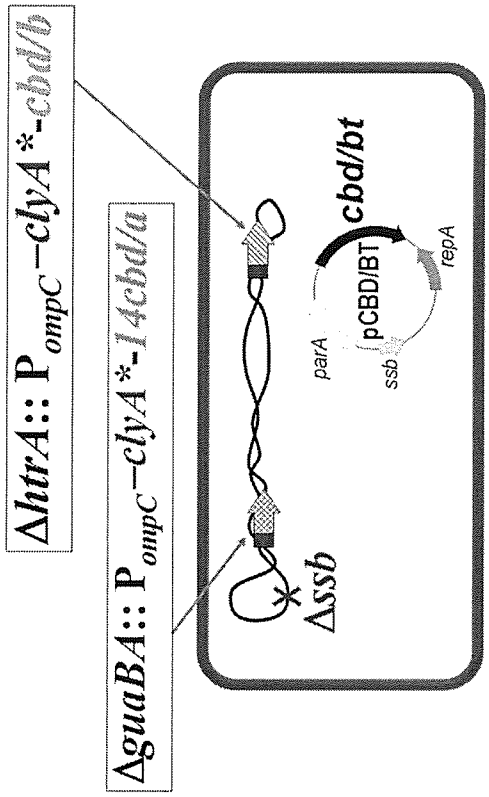


Fig. 10


ΔhtrA:: P ompC –clyA*-cbd/b
ΔguaBA:: P ompC –clyA*-14cbd/a
Δssb
parA
pCBD/BT
cbd/bt
ssb
repA