RECOMBINANT ATTENUATED CLOSTRIDIUM ORGANISMS AND VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a non-provisional application that claims priority under 35 U.S.C. § 119(e) of provisional applications U.S. Serial No. 60/792,553 filed April 17, 2006, the contents of which are hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention is related to attenuated *Clostridium* organisms, methods of making and using the same, and mutein *alpha*-toxins and nucleic acids encoding the same.

BACKGROUND OF THE INVENTION

Anaerobic bacterial pathogens are a serious economic burden on the agricultural industry. Bacteria of the *Clostridium* family represent a particular burden, because these bacteria cause serious diseases in poultry and other economically valuable domestic animals. Previous efforts to control these organisms have relied upon sanitary measures and the administration of antibiotics in the animal feed.

In particular, *Clostridium perfringens* ("*C. perfringens*") is an anaerobic bacterium that is found in the soil, decaying organic matter, and as part of the gut flora of humans and animals. Different strains of *C. perfringens* are designated as biotypes A through E, depending on the spectrum of toxins produced [Justin *et al.*, *Biochemistry 41*, 6253-6262 (2002); McDonel (1986) PHARMACOLOGY OF BACTERIAL TOXINS; F Dorner and J Drews (Eds.) Pergamon Press, Oxford]. Biotype A strains are of particular importance as the etiological agents of various types of gangrene and enteric diseases. A particularly serious enteric disease caused by *C. perfringens* is enteritis necroticans (also art-known as, "necrotic enteritis'), a gangrene of the intestines resulting in necrosis, sepsis, and hemolysis, in both humans and domesticated animals [*see*, Pearson *et al.*, *J. Am. Vet. Med. 188(11)*:1309-10 (1986); Al-Sheikhy and Truscott, *Avian Dis. 21(2)*:256-63 (1977)]. For avians, *e.g.*, chickens (*Gallus gallus*), enteritis necroticans is a significant problem. *C. perfringens* of either type A or type C can cause major losses, especially in production broiler chickens [Ficken and Wages, *Necrotic Enteritis*, In Diseases of Poultry, 10th Ed. pps 261-264 (1997)]. In addition to losses associated with necrotic enteritis outbreaks, productivity is reported to be

impaired in flocks with *C. perfringens*-associated disease [Lovl and Kaldhusdal, *Avian Pathology* 30:73-81 (2001)]. As noted above, antibacterial agents inserted in the animal feed are the most common method of control. However, antibacterial agents, *e.g.*, antibiotics, are costly and subject to increasing concerns related to the promotion of bacterial resistance.

More recently, attempts have been made to provide vaccines against harmful *Clostridium* species. For example, Lovland *et al.* [*Avian Pathology 33(1)*:83-92 (2004)] demonstrated candidate vaccines based on *C. perfringens* type A and type C toxoids with an aluminum hydroxide adjuvant. Vaccination of parent hens was reported to provide specific antibodies to protect progeny against enteric lesions induced by subclinical challenge with *C. perfringens*. Other toxoid-based vaccines prepared from detoxified *C. perfringens* toxins are known [*see e.g.*, U.S. Patent No. 4,292,307, which describes toxoids of *C. perfringens* types A, B and D, *Cl. oedematiens*, and *Cl. septicum*].

Recombinant toxoid preparations also have been proposed. For example, Titball *et al.*, [U.S. Patent Nos. 5,851,827, 6,403,094, and 5,817,317] report nucleic acids that encode antigenic *C. perfringens* peptides, as well as the peptides themselves, and vaccines prepared from the peptides. Peptides are described for example, which have amino acid residues 261 to 300 of the natural *C. perfringens alpha*-toxin, but lack the phosphoplipase C and sphinogmyelin hydrolyzins domains of the natural toxin. It was further reported that these peptides induce immune protection against the natural toxin. In addition, U.S. Patent No. 6,610,300 describes a vaccine based on an antigenic fragment of a mutein *C. perfringens beta*-toxin.

However, no matter whether a toxoid vaccine is derived from the native organism or is obtained recombinantly, it is considered to be economically burdensome to produce and administer toxoid proteins to animals in need of immunization, except under special circumstances (*e.g.*, treating humans who might be allergic or sensitive to other components of a whole organism vaccine). Further, protein/toxoid based-vaccines typically require repeated booster vaccinations in order to maintain full effectiveness.

Another proposed solution has been to engineer an antigenically active virus that will produce a mutein *alpha*-toxin, in place of the wild-type toxin. For example, Bennett *et al.* [*Viral Immunol. 12(2)*:97-105 (1999)] have demonstrated a recombinant vaccinia virus vector that expresses a nontoxic C-domain of *C. perfringens alpha*-toxin.

Unfortunately, while several recombinant vaccinia vaccines have been proposed during the past 20 years, there are still longstanding concerns about the safety of releasing live, infectious vaccinia viruses into an environment where they might be transmitted to those people who are not resistant to this virus.

The *alpha*-toxin (*plc* gene) of *C. perfringens* is known to possess several biological activities including hemolytic activity, phospholipase C, sphingomyelinase, phosphodiesterase, and lethal activities. There are a number of reports in the art concerning mutations to this *alpha*-toxin that reduce toxicity. Schoepe, *et al. [Infect. and Immun. 69(11)*: 7194-7196 (2001)] describe a naturally-occurring *C. perfringens* strain that produces a non-toxic *alpha*-toxin. However, it would be difficult to modify this strain to elicit immune protection against other variant, but toxic wild-type *C. perfringens* species.

Williamson and Titball [*Vaccine 11(12)*:1253-1258 (1993)] showed that the region of the toxin from amino acid residues 247 to 370 alone was sufficient to immunize mice against gas gangrene experimentally induced by C. perfringens. Alape-Girón *et al.* [*Eur. J. Biochem. 267*:5191-5197 (2000)] have reported that substitutions in Asp269, Asp336, Tyr275, Tyr307, and Tyr331 reduced *alpha*-toxin toxicity. Nagahama, *et al.* [*Infect. and Immun. 65*:3489-3492 (1997)] reported that replacement of Asp-56, Asp-130, or Glu-152 resulted in reduced *alpha*-toxin toxicity. Nagahama *et al.* [*J. Bacteriology 177*:1179-1185 (1995)] reported that substitution of the histidine at position 68 with a neutral amino acid, such as glycine, in the *C. perfringens alpha*-toxin resulted in a complete loss of hemolytic, phospholipase C, sphingomyelinase, and lethal activity of the mutein *alpha*-toxin. This single amino acid change was believed to inactivate one of the three zinc-binding domains of the protein. The zinc-binding domain inactivated by substitution of His68 was later denoted as Zn2 [Justin *et al.*, *Biochemistry 41*:6253-6262 (2002)].

Despite the foregoing, there remains a need in the art for a safe, economical and effective method of protecting intensively cultivated domestic animals, including avians, such as chickens, from infection by *Clostridium* species, including *C. perfringens*.

The citation of any reference herein should not be construed as an admission that such reference is available as "prior art" to the instant application.

SUMMARY OF THE INVENTION

In order to address the above-described shortcomings in the art, the present

invention provides nucleic acid molecules that encode a substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin. In one such embodiment, the nucleic acid molecule encodes a mutein *alpha*-toxin that comprises the amino acid sequence of SEQ ID NO: 3, minus at least 18 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In another embodiment, the nucleic acid molecule encodes a mutein *alpha*-toxin that comprises the amino acid sequence of SEQ ID NO: 3, minus at least 12 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In still another embodiment, the nucleic acid molecule encodes a mutein that comprises the amino acid sequence of SEQ ID NO: 3 minus at least 9 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In yet another embodiment, the nucleic acid molecule encodes a mutein that comprises the amino acid sequence of SEQ ID NO: 3 minus at least 6 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In still another embodiment, the nucleic acid molecule encodes a mutein that comprises the amino acid sequence of SEQ ID NO: 3 minus at least 3 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}.

In one embodiment, a nucleic acid molecule of the present invention encodes a mutein in which no more than 48 consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3. In another embodiment, a nucleic acid molecule encodes a mutein in which no more than 36 consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3. In yet another embodiment a nucleic acid molecule encodes a mutein in which no more than 24 consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3. In still another embodiment a nucleic acid molecule of the present invention encodes a mutein in which no more than 18 consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3.

In a particular embodiment, the present invention provides a nucleic acid molecule encoding a mutein in which nine consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3, one of which is His_{68}. In a particular embodiment of this type, the nucleic acid molecule encodes a mutein in which the deleted nine consecutive amino acid residues range from Tyr_{62} through Trp_{70} of SEQ ID NO: 3. In a more particular embodiment, the nucleic acid molecule encodes a mutein in which these deleted nine consecutive amino acids are replaced by a single leucine residue.

In another embodiment, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 2, wherein nucleotides 268-294 are deleted. In a specific embodiment of this type, nucleotides 268-294 of the nucleotide sequence of SEQ ID NO:2 are replaced by three nucleotides that encode a single leucine residue.

The invention also provides substantially nontoxic muteins of *Clostridium perfringens alpha*-toxin. In one such embodiment the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3 minus at least 18 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In another embodiment, the mutein comprises the amino acid sequence of SEQ ID NO: 3 minus at least 12 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In still another embodiment, the mutein comprises the amino acid sequence of SEQ ID NO: 3 minus at least 9 consecutive amino acid residues, in which one of the deleted amino acid residues is His_{68}. In yet another embodiment, the mutein comprises the amino acid sequence of SEQ ID NO: 3 minus at least 6 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}. In still another embodiment, the mutein comprises the amino acid sequence of SEQ ID NO: 3 minus at least 3 consecutive amino acid residues, wherein one of the deleted amino acid residues is His_{68}.

In one embodiment, a mutein of the present invention comprises no more than 48 consecutive amino acid residues that are deleted from the amino acid sequence of SEQ ID NO: 3. In another embodiment, the mutein comprises no more than 36 consecutive amino acid residues deleted from the amino acid sequence of SEQ ID NO: 3. In yet another embodiment the mutein comprises no more than 24 consecutive amino acid residues deleted from the amino acid sequence of SEQ ID NO: 3. In still another embodiment the mutein comprises no more than 18 consecutive amino acid residues deleted from the amino acid sequence of SEQ ID NO: 3.

In a particular embodiment, the present invention provides a substantially nontoxic mutein in which nine consecutive amino acid residues are deleted from the amino acid sequence of SEQ ID NO: 3, one of which is His_{68}. In a more particular embodiment of this type, the deleted nine consecutive amino acid residues range from Tyr_{62} through Trp_{70} of SEQ ID NO: 3. In still a more particular embodiment, these deleted nine consecutive amino acids are replaced by a single leucine residue in the amino acid sequence of the mutein.

The invention further provides attenuated *Clostridium perfringens* organisms that

have a nucleic acid molecule that encodes a substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin integrated into their chromosomes. This integrated nucleic acid molecule is preferably located at a position on the chromosome that is homologous to the location of the nucleic acid molecule that encodes the wild-type *alpha*-toxin in the wild-type *Clostridium perfringens* organism. Thus, an attenuated *Clostridium perfringens* organism of the present invention can be substantially nontoxic due to the lack of a functional wild-type *plc* gene. As exemplified herein, the attenuated *Clostridium perfringens* organism can be a type A *Clostridium perfringens*.

In a particular embodiment of the present invention, the attenuated *Clostridium perfringens* organism is Clostridium *perfringens* CPERF/ΔαToxin 365-054 (ATCC Deposit No. PTA7364). In another particular embodiment of the present invention, the attenuated *Clostridium perfringens* organism is Clostridium *perfringens* CPERF/ΔαToxin 365-053 (ATCC Deposit No. PTA7365).

A *Clostridium perfringens* organism that is attenuated by the methods of the present invention can be isolated from a host animal that is either a mammal or an avian. Such mammals can include: bovine, ovine, and porcine. Examples of appropriate avians include chickens, turkeys, ducks, pigeons, geese, doves, swans, partridge, and grouse.

The present invention also provides vaccines. Such vaccines can comprise the attenuated *Clostridium perfringens* organisms of the present invention. The vaccines of the prevent invention can also include pharmacologically acceptable buffers, excipients, and/or adjuvants.

In addition, the invention provides methods of inducing immunity to *Clostridium perfringens* in an animal. One such embodiment comprises administering an immunologically effective dose of a vaccine of the present invention to the animal. The vaccines of the present invention can be administered by a number of routes including: orally, intramuscularly, intravenously, intradermally, subcutaneously and intranasally. A vaccine of the present invention can be top-dressed on the feed of the animal and/or sprayed onto the animals to provide for oral administration. The present invention further provides an animal feed that includes a vaccine of the present invention.

The present invention also provides an attenuated *Clostridium perfringens* organism of the present invention that in addition, expresses at least one gene encoding a non-*Clostridium perfringens* polypeptide. In one such embodiment one or more of the non-*Clostridium perfringens* polypeptides are bacterial polypeptides, such

as antigenic proteins from *E. coli,* salmonella, lawsonia, or campylobacter etc., and/or combinations thereof. Alternatively, or in combination therewith, a non-*Clostridium perfringens* polypeptide can be a non-bacterial polypeptide. Examples of such non-bacterial polypeptides include mammalian or avian proteins, *e,g.* cytokines, such as chicken IL-18; viruses such as rotavirus or coronavirus; and parasites such as eimeria, isospora, and cryptosporidium.

In another aspect, the present invention provides an antibody that selectively binds to an epitope missing from the substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin. Such antibodies can distinguish the substantially nontoxic mutein from a wild-type *Clostridium perfringens alpha*-toxin.

Test kits are also provided that include the antibodies of the present invention for use in identifying whether a subject animal has been vaccinated, or alternatively, has been naturally infected by a *Clostridium perfringens* organism.

Accordingly, methods of identifying and/or distinguishing an animal that has been naturally infected by a *Clostridium perfringens* organism from one vaccinated with an attenuated *Clostridium perfringens* organism are also provided. In one such embodiment the method entails contacting a fluid sample from the animal with an antibody that selectively binds to an epitope found in a wild type *Clostridium perfringens alpha*-toxin that has been deleted from the substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of the present invention. Therefore, the antibody can distinguish those animals that have been vaccinated with a substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of the present invention, (and/or an attenuated *Clostridium perfringens* organism expressing the mutein) from those that are infected or had been infected by a wild type *Clostridium perfringens alpha*-toxin. The next step is to determine whether the antibody reacts with the fluid sample, *e.g.*, binds to an antigen contained by the fluid sample. The animal is identified as one that has/had been naturally infected by a *Clostridium perfringens* organism when the antibody reacts with the fluid sample.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates a genomic map of the *C. perfringens alpha*-toxin encoding region. The locations of the two large (1182 base pair and 1746 base pair) fragments, respectively, that were used to construct CPERF001 are shown. The location of the resulting 27 base pair deletion is also indicated. "*Yplc*" indicates the *yplc* gene (CPE0035); "plc"

indicates the gene encoding *alpha*-toxin (a phospholipase C), and "CobW" indicates a downstream gene.

FIG. 2A illustrates the sequence of a portion of the plc gene from *C. perfringens* strain CP6 [SEQ ID NO: 4; this is a portion of SEQ ID NO: 22, (*i.e.*, nucleotides 262-300) of a *plc* gene fragment from CP6] and the corresponding peptide sequence (SEQ ID NO: 5), where the underlining indicates the BamH1 endonuclease restriction site used in creating the deletion.

FIG. 2B illustrates the sequence of the primer (SEQ ID NO: 6) used to create the deletion in the parent *C. perfringens* strain 1240, giving rise to the deletant CPERF001. The underlining indicates the BamH1 restriction site included in the primer to facilitate construction of the deletion. Also illustrated is the corresponding peptide (SEQ ID NO: 7).

FIG. 2C illustrates the sequence of the resulting deletion in CPERF001 (SEQ ID NO: 8; nucleotides 103-117) and in the corresponding peptide (SEQ ID NO: 9). The underlining indicates the restored BamH1 endonuclease restriction site.

FIG. 3A once again illustrates the sequence of a portion of the plc gene from *C. perfringens* strain CP6 [SEQ ID NO: 4, nucleotides 262-300] and the corresponding peptide sequence (SEQ ID NO: 5), where the underlining indicates the BamH1 endonuclease restriction site used in creating the deletion.

FIG. 3B illustrates the sequence of the primer (SEQ ID NO:10) used to create the deletion in the parent *C. perfringens* strain 29, giving rise to the deletant CPERF002. Also illustrated is the corresponding peptide (SEQ ID NO:11).

FIG 3C illustrates the sequence of the resulting deletion in CPERF002 (SEQ ID NO: 12) and corresponding peptide (SEQ ID NO: 13).

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the invention provides modified *Clostridia* organisms and cultures that express one or more of the *Clostridia* toxins, *e.g.*, *alpha*-toxins, as muteins that have no detectable toxicity, and/or substantially low toxicity, relative to the native or wild-type *Clostridia* toxins. Advantageously, the inventive *C. perfringens* mutant organisms are readily administered as a live vaccine to animals. The inventive mutein *C. perfringens alpha*-toxins are also provided, together with nucleic acid molecules that

encode the muteins, vectors for expressing the *alpha*-toxins, and methods of using the same.

In order to provide a clear description of the invention, several terms are defined, as follows. A vaccine is a composition that includes an immunogen, and other optional pharmaceutically acceptable ingredients, including, in certain embodiments, suitable adjuvants. As used herein, the term "immunogen" describes a composition, substance or vector, that when introduced into an animal, stimulates an immune response. For purposes of the present invention, an immunogen is contemplated to include any vector capable of expressing or introducing the inventive mutein *alpha*-toxin into an animal to be immunized. A vector includes, *e.g.*, the inventive *C. perfringens* or other suitable microorganism, that expresses the inventive mutein *alpha*-toxin when the vector is introduced into an animal. A vector also includes art-known nucleic acid molecules, *e.g.*, plasmids and the like, that express the inventive mutein *alpha*-toxin when directly introduced into an animal, *e.g*, by entering a cell of the animal and expressing the mutein *alpha*-toxin in the animal. An immunogen is also a protein, such as the inventive *alpha*-toxin, employed by itself or as part of a suitable vaccine composition.

As used herein, and unless otherwise specified, the terms "immunize" and "vaccinate" are synonymous and are used interchangeably to describe the introduction of an immunogen into an animal to elicit an immune response in the animal. The elicited immune response provides protective immunity to the treated animal that limits or reduces clinical disease signs, *e.g.*, gas gangrene, and/or mortality, in vaccinated animals that are later challenged with a virulent dose of a *C. perfringens* species for which the inventive vaccine is protective.

The term "adjuvant" is defined as one or more substances that cause stimulation of the immune system. In this context, an adjuvant is used to enhance an immune response to one or more vaccine antigens/isolates. An adjuvant may be administered to the target animal before, in combination with, or after the administration of the vaccine. Adjuvants of the present invention may be obtained from any of a number of sources including from natural sources, recombinant sources, and/or be chemically synthesized, etc. Examples of chemical compounds used as adjuvants include, but are not limited to aluminum compounds; metabolizable and non-metabolizable oils; block polymers; ISCOM's (immune stimulating complexes); vitamins and minerals (including but not limited to: vitamin E, vitamin A, selenium, and vitamin B12); Quil A (saponins); crosslinked acrylic acid-based polymers (*e.g.*, prop-2-enoic acid polymers) crosslinked

to different levels with a polyalkenyl polyether, as sold under the trademark CARBOPOL®; and/or uniformly dispersed micron size oil droplets in water emulsion, *e.g.*, as sold under the trademark Emulsigen®.

Additional examples of adjuvants, that sometimes have been referred to specifically as immune stimulants, include, bacterial and fungal cell wall components (*e.g.*, lipopolysaccarides, lipoproteins, glycoproteins, muramylpeptides, *beta*-1,3/1,6-glucans), various complex carbohydrates derived from plants (*e.g.*, glycans, acemannan), various proteins and peptides derived from animals (*e.g.*, hormones, cytokines, co-stimulatory factors), and novel nucleic acids derived from viruses and other sources (*e.g.*, double stranded RNA, CpG). In addition, any number of combinations of the aforementioned substances may provide an adjuvant effect, and therefore, can form an adjuvant of the present invention.

The term "antibody" as used herein is intended to encompass polyclonal antibodies, monoclonal antibodies, and/or fragments or recombinant derivatives thereof, including engineered binding proteins incorporating antibody variable domains.

As used herein, the residue numbering and position of the amino acid residues of the inventive *alpha*-toxin proteins are based on the number system described for *Clostridium perfringens* strain 13. The *C. perfringens* strain 13 *alpha*-toxin is reported by GenBank Accession No. NC 003366 as illustrated by SEQ ID NO: 1. The entire protein is 398 amino acids long. The *alpha*-toxin encoded by the mutein vectors exemplified hereinbelow correspond to the protein of SEQ ID NO: 1 having a deletion of amino acid residues 90-98. There is a 28 amino acid signal sequence which is cleaved off during the maturation of the protein. Therefore the exemplified deletion corresponds to amino acid residues 62-70 of the mature protein, that is 370 amino acids in length (SEQ ID NO: 3).

The codon numbering of the DNA encoding the inventive *alpha*-toxin proteins is based upon the *plc* gene of *Clostridium perfringens* strain 13, as reported in GenBank Accession No. NP 560952, and as illustrated by SEQ ID NO: 2. The coding sequence for *alpha*-toxin runs from nucleotide 48590 through 49786 in *C. perfringens* strain 13. The codon deletion in the two constructs exemplified herein corresponds to nucleotides 48857 through (and including) 48883 of the NP 560952 gene. This deletion is found within the *alpha*-toxin gene and corresponds to nucleotides 268-294 in the coding sequence of SEQ ID NO: 2.

Further, the use of singular terms for convenience in description is in no way

intended to be so limiting. Thus, for example, reference to a composition comprising a *C. perfringens* cell includes reference to one or more of such cells. It is also to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

In a particular aspect of the present invention, non-reverting mutants of *C. perfringens* are provided that are "substantially nontoxic," *i.e.*, organisms that express an immunogenic *alpha*-toxin of little or no toxicity thereby rendering them suitable for use as protective vaccines. The inventive *C. perfringens* organisms therefore bestow a sufficiently reduced toxicity, relative to wild-type *C. perfringens* organisms, to render them tolerable as a vaccine or antigen, when employed under conditions effective to elicit an anti-*alpha*-toxin or anti-*C-perfringens*, immune response in an animal that is so vaccinated. Thus, the inventive *C. perfringens* organism is "attenuated" relative to the wild-type *C. perfringens*.

The phrase, "substantially nontoxic" is also intended to apply to the above-noted immunogenic *alpha*-toxin muteins that have sufficiently low or no toxicity, thereby also making them suitable for use in a protective vaccine.

The reduction in toxicity is measured, *e.g.*, by one of the following art-known tests: hemolytic activity, phospholipase C activity, sphingomyelinase activity, phosphodiesterase activity, and general lethal activity in a test animal group. Generally, no residual toxicity is detectable by such standard tests. Nevertheless, the presence of a minimal level of one or more of these activities, *e.g.*, from about 10^{-4} to about 10^{-2}, relative to the toxicity of an equivalent number of infectious units of the wild-type *C. perfringens alpha*-toxin from which the mutein has been derived, may prove acceptable in a veterinary setting.

In a one embodiment, the invention is practiced employing biotype A strains of *C. perfringens*, which are of particular importance as the etiological agents of various types of gangrene and enteric diseases. In particular, the *alpha*-toxin of *C. perfringens* is the target for deletion-attenuation, since attenuation of this toxin is sufficient to render *C. perfringens* sufficiently non-lethal, relative to wild-type strains.

Broadly, the inventive *C. perfringens plc* gene expresses a mutein *alpha*-toxin.

The mutein alpha-toxin has a deletion that includes the His of the Zn2 loop, together with flanking residues, in order to greatly reduce the possibility of any back mutation to the toxic form. It has now been found that deletion of the Zn2 loop His residue, *e.g.*, His_{68} of SEQ ID NO: 3, together with the deletion of additional residues flanking the His residue of the Zn2 loop, provides an *alpha*-toxin retaining sufficient immunogenicity to induce protective immunity in animals vaccinated with the inventive *C. perfringens*, while also being unlikely to undergo a back-mutation to encoding a wild-type *alpha*-toxin. The additional residues that can be deleted are deleted in the C-terminal direction and/or in the N-terminal direction, relative to His_{68}, and can range in number from about 4 through about 60 residues in either of those directions. Alternatively, His_{148} and flanking residues can be similarly deleted.

One embodiment of the inventive mutein *alpha*-toxin also includes, in addition to the deletion of His_{68}, a deletion of at least 30 amino acid residues from either side (in the C-terminal or N-terminal direction) of the His_{68} position, relative to SEQ ID NO: 3. In another embodiment, the inventive mutein *alpha*-toxin includes, in addition to the deletion of His_{68}, a deletion of at least 20 amino acid residues from either side of His_{68}, relative to SEQ ID NO: 3. In still another alternative embodiment, the inventive mutein *alpha*-toxin includes, in addition to the deletion of His_{68}, a deletion of at least 5 amino acid residues from either side of His_{68}, relative to SEQ ID NO: 3. In yet another embodiment, the inventive mutein *alpha*-toxin includes a deletion from about residue 62 through about residue 70, relative to SEQ ID NO: 3. Optionally, the deleted amino acid residues are replaced by one or more other residues, such as a single leucine residue.

In still further another embodiment, the mutein *C. perfringens alpha*-toxin is produced and isolated from *C. perfringens*, or from an alternative recombinant organism, to be employed as a research reagent, and/or in diagnostic kits or assays, *e.g.*, as a target for anti-*alpha*-toxin antibodies. Yet another utility for the mutein *C. perfringens alpha*-toxin proteins is in specialized vaccines for animals, *e.g.*, humans, that may not tolerate vaccination with the inventive attenuated *C. perfringens* organism.

In addition, the present invention provides an antibody that specifically binds to the wild-type *alpha*-toxin relative to the *alpha*-toxin muteins of the present invention.

In a further embodiment, an antibody is provided that preferentially binds to wild-type *C. perfringens alpha*-toxin protein while exhibiting minimal or no binding to the inventive mutein *alpha*-toxin, *e.g.*, avoiding binding to an *alpha*-toxin mutein that has a deletion as described in detail, *supra*. The antibody provided is therefore useful for

distinguishing the deletion mutein *alpha*-toxin from the wild-type *alpha*-toxin, and thereby, also useful for distinguishing animals vaccinated with a vaccine of the present invention from animals that have been infected by wild type *C. perfringens*. Methods of eliciting and screening for such selective antibodies are art-known. Similarly, antibodies that recognize the inventive mutein *alpha*-toxin of the present invention, but not the wild-type protein can also be generated. The antibodies of the present invention can be polyclonal, monoclonal ("mAb") or fragments or engineered fragments or derivatives of such antibodies retaining selective binding properties.

Techniques for preparing and screening monoclonal antibodies have been amply described [*see, e.g.,* Stites, *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.), Lange Medical Publications, Los Altos, California (1988); Harlow and Lane, *Antibodies: A Laboratory Manual*, CSH Press (1988); Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.), Academic Press, New York (1986); and Kohler and Milstein, *Nature* 256:495-497 (1975), all of which are incorporated by reference herein in their entireties].

For instance, and without limitation, an immune response is elicited in suitable animals, such as a mouse or chicken, by vaccination with a purified wild-type *C. perfringens alpha*-protein, *e.g.*, in combination with a suitable adjuvant. For example, in order to avoid the toxicity of the wild-type *alpha* protein, the immunogen is a peptide corresponding to the deleted residues, and, if necessary the immunogenicity of the peptide is enhanced by combination with a suitable adjuvant or by coupling to a suitable carrier protein. Coupling to a carrier protein is art known, and can be accomplished, *e.g.*, by providing the peptide with a terminal cysteine, and coupling it to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) using either maleimide coupling chemistry or sulfosuccinimidyl 4-[N maleimidomethyl]cyclohexane-1-carboxylate) linker (from Pierce) A carbodiimide linker also may be employed without requiring a terminal cysteine.

For preparation of monoclonal antibodies, splenic lymphocytes can be obtained from an immunized animal, hybridomas are prepared from those lymphocytes, and one or more potentially suitable hybridomas expressing anti-*alpha* protein can be obtained. The hybridomas are screened against both mutein and wild-type *alpha*-protein, and a hybridoma expressing an antibody that binds only to the wild-type *alpha*-toxin is identified, cloned and employed to produce monoclonal antibodies that bind only to the wild-type *alpha* protein. Optionally, cDNA from the identified hybridoma clonal line is

obtained, and recombinant antibodies or antibody fragments can be produced in other art-known expression systems.

As discussed above, one potential disadvantage of vaccinations in general is that the resulting vaccinated animals can generate false positives when testing for infection using antibodies raised against a naturally occurring strain, thereby, hindering the identification of infected animals. Therefore, the present invention provides a test kit for distinguishing a subject animal that has been infected by a naturally occurring *C. perfringens* organism from one vaccinated with a mutein *alpha*-toxin of the present invention.

One such test kit includes a quantity of a selective anti-wild-type *alpha*-toxin antibody that exhibits minimal or no binding to a mutein *alpha*-toxin of the present invention. The kit can also include other suitable reagents, sufficient for conducting at least one diagnostic test. In a further embodiment, the antibody is tagged or labeled with a readily detectable maker moiety, *e.g.*, any art-known enzymatic marker, *e.g.*, peroxidase; fluorescent tag, *e.g.*, fluorescein; beads, including magnetic beads; and the like. Optionally, the kit can further include a tagged antibody that selectively binds to the selective anti-wild-type *alpha*-toxin antibody. Immunoassays are well known to the art, and include sandwich immunoassay, competitive immunoassays, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA) and others.

Also provided are methods for identifying and distinguishing an animal that has been infected by a naturally occurring, *i.e.*, wild-type, *C. perfringens* organism, from an animal vaccinated with a vaccine comprising the attenuated *C. perfringens* organism of the invention, that is conducted, for example, by the following steps:

(a) contacting a fluid sample from the animal with an above-described selective anti-wild-type *alpha*-toxin antibody that exhibits minimal or no binding to a mutein *alpha*-toxin of the present invention; and

(b) determining whether the antibody reacts with the fluid sample;

wherein when the antibody reacts with the fluid sample, the animal is identified as one that has been infected by a naturally occurring *C. perfringens* organism.

Producing Attenuated *C. Perfringens* Strains

A process of converting a wild-type *C. perfringens* isolate into an attenuated, or substantially nontoxic, strain suitable to be administered as a vaccine can be conducted as follows. The *alpha*-toxin (*plc*) gene can be replaced on the bacterial

chromosome with a gene encoding only an *alpha*-toxin mutein, leaving no remaining capability for the *C. perfringens* organism to produce wild-type *alpha*-toxin. Very broadly, the process of producing the vaccine organism includes, but is not limited to, the following general steps, not necessarily in the order presented.

(1) Identifying the type of animal to be protected by vaccination and one or more clinical isolates obtained for screening purposes. This step is typically optional in the case for the *alpha*-toxin, since isolates from one species of animal are more likely than not to provide protection for other species of animal.

(2) Amplifying the *plc* gene from the *C. perfringens* isolate or isolates, *e.g.,* by PCR or other art-known nucleic acid amplification technique, with suitable flanking primers, and employing amplification with suitable primers to create the desired deletion mutation. Alternatively, an appropriate library can be probed.

(3) Creating a suicide vector comprising the deletion *plc* gene, in which the *C. perfringens* origin of replication has been removed, and/or an origin of replication that can replicate in *C. perfringens* is simply not present; and in either case, including suitable selectable markers, *e.g.*, antibiotic markers, adjacent to the mutated *plc* gene. Such a vector can then be inserted into *C. perfringens* organisms, *e.g.*, by electroporation, or other art-known methods.

(4) Selecting *C. perfringens* organisms in which the mutein *plc* gene has been successfully integrated into the bacterial chromosome. This is done by culturing the *C. perfringens* organisms of step (3) in the presence of the selectable agent, *e.g.*, an antibiotic(s) corresponding to the selectable markers. For example, the only *C. perfringens* organisms that would grow under antibiotic selection would be those that through homologous recombination have directly integrated the suicide vector, with its antibiotic resistance gene(s), into the bacterial chromosome. These growing *C. perfringens* organisms therefore, would have two adjacent *plc* genes, one being wild-type, whereas the other would have the deletion mutation.

(5) Selecting *C. perfringens* organisms that have undergone a further recombination event that removes the selectable markers, *e.g.*, antibiotic markers, along with the wild-type *plc* gene. This is done by culturing the organisms of (4) in the absence of the selectable agent, *e.g.*, the antibiotic, and selecting for non-hemolytic clones on blood agar.

Since the insertion of the mutein nucleic acid is accomplished by homologous recombination, the nucleic acid molecule encoding the mutein *alpha*-toxin is incorporated at a chromosomal position that is homologous to the location of a nucleic acid molecule encoding a wild-type *alpha*-toxin that is present in non-attenuated *Clostridium perfringens*.

In more detail, field isolates of *C. perfringens* are obtained from diseased animals or other sources. Initially, genomic DNA obtained from field isolates of interest is inserted into a suitable dual microorganism shuttle vector, *e.g.*, a shuttle plasmid with selectable markers, *e.g.*, antibiotic markers, to assess their transformability. Broadly, a suitable shuttle vector will include one, two, three or more of the following features, a cloning site, a *C. perfringens* origin of replication, an *E. coli* origin of replication, and an antibiotic resistance gene andor selectable marker. Art-known vectors suitable for this purpose, or readily adaptable for this purpose include, for example, the recombinant shuttle plasmid pHR106 described by Roberts *et al.*, [*Appl Env Mircobiol 54*: 268-270 (1988)]; the pJIR 750 and pJIR 751 plasmids described by Bannam, *et al.*, [*Plasmid 29:*233-235 (1993)]; the promoterless pPSV promoter selection vector of Matsushita, *et al.*, 1994, *Plasmid 31,* 317-319; the shuttle plasmids pJIR1456 and pJIR1457, described by Lyras, *et al.*, 1988, *Plasmid 39,* 160-164; and the pAK201 shuttle vector described by Kim *et al.*, 1989, *Appl Environ Microbiol 55,* 360-365, the contents of which are incorporated herein by reference in their entireties. Removal of the *C. perfringens* origin of replication converts the shuttle vector into a suicide vector.

For example, one shuttle plasmid is pJIR418, described by Sloan, *et al.*, 1992, *Plasmid* 27, 207-219, incorporated by reference herein.

Isolates yielding $\geq 10^4$ transformants per microgram of plasmid DNA, and that are susceptible to the antibiotic marker, *e.g.*, chloramphenicol or erythromycin, are potential candidates for deletion. Genomic DNA from the candidate strains is then used as a template for long range PCR of the *plc* (*alpha*-toxin) gene and flanking sequences of the candidate strains. For example, as exemplified hereinbelow, the *C. perfringens* strain 13 chromosome was used to identify primers for amplifying the gene encoding the *alpha*-toxin. These primers were then used to clone the *alpha*-toxin gene from another strain, that was the CP6 poultry isolate.

After subcloning of the PCR products, the *alpha*-toxin gene and flanking regions are sequenced and restriction mapped. New oligonucleotide primers are synthesized with flanking restriction sites and the products of two separate amplifications are cloned

into a suitable suicide plasmid (*C. perfringens* origin of replication has been removed), *e.g.*, exemplified hereinbelow as plasmid 1192-23.1 to create the desired vaccine strain with the deletion.

The provided suicide vector(s) specific to the isolate(s) are inserted into the corresponding animal strain of *C. perfringens*, by any standard art-known method. For example, this is accomplished by electroporation. When the suicide vector is inserted into *C. perfringens* (without the *C. perfringens* origin of replication, it is unable to replicate in the cytoplasm, and does not survive unless it successfully integrates into the bacterial chromosome.) Successful integrants are the only organisms that will grow in the presence of the antibiotic corresponding to the newly introduced antibiotic marker gene.

Any art-known selectable marker gene may be employed, although choramphenicol and/or erythromycin markers are employed in the vectors exemplified hereinbelow.

These recombinant events result from homology of the wild-type *plc* gene to the deletion *plc* gene plasmid DNA. The resulting recombinant bacteria is termed an integrant. The integrant contains a copy of the introduced homology vector that is integrated at the *plc* gene locus. Thus, the resulting integrant includes two copies of the *plc* gene, the original normal copy, and the introduced deleted version. The introduced antibiotic resistance genes are located between the two copies of the *plc* gene. Rare random recombination events can occur between the two copies of the *plc* gene. This recombination event can produce one of two outcomes. In both cases, the DNA intervening between the two copies of the *plc* gene (including the resistance genes) has been removed. In the first outcome, the normal or wild-type *plc* gene is restored, resulting in recovery of the original parent strain without antibiotic markers. In the second outcome, the wild-type *plc* gene is replaced by the deleted copy, producing the desired *alpha*-toxin deletant construct, without the antibiotic markers.

Removing the antibiotic from the culture medium allows those bacteria that have undergone recombination to survive and replicate. The deletion recombinant clones are then identified by growth on blood agar, without the hemolysis normally exhibited by *C. perfringens* that expresses the wild-type *alpha*-toxin.

Animals to be Vaccinated

Animals for which an attenuated *C. perfringens* vaccine can be produced, and

from which a useful *C. perfringens* wild-type strain may be isolated, include, broadly, any animals for which *C. perfringens* infection is a problem. Vertebrates of interest include avians, mammals, and fish, and particularly animals of economic and/or agricultural importance. The following list of animals are those that are contemplated to benefit from a *C. perfringens* vaccine and/or from which useful *C. perfringens* wild-type isolates may be obtained. While it is sometimes possible that any such vaccine comprise a component (living or non-living) that was originally isolated from the same genus or species of animal that is to be vaccinated, this is not a requirement.

A non-limiting list of such animals include those of the avian, bovine, ovine, etc., families, as well as aquatic animals, *e.g.*, that may be subjected to aquaculture and/or harvested from the wild and kept alive in holding tanks for a time prior to marketing. These include fish such as trout or salmon, and other species raised or harvested for economic benefit. Non-vertebrate aquatic animals include lobsters, crabs, mollusks, *e.g.*, squid, octopus, clams, oysters, muscles, scallops, and the like. Avian shall be understood to include, for example, chickens, turkeys, geese, duck, etc. Bovine shall be understood to include, for example, cattle, beef, veal, etc. Ovine shall be understood to include, for example, lamb, etc.

For purposes of the present invention, the term "fish" shall be understood to include without limitation, the *Teleosti* grouping of fish, *i.e.*, teleosts. Both the *Salmoniformes* order (which includes the *Salmonidae* family) and the *Perciformes* order (which includes the *Centrarchidae* family) are contained within the *Teleosti* grouping.

Examples of potential fish recipients include the *Salmonidae* family, the *Serranidae* family, the *Sparidae* family, the *Cichlidae* family, the *Centrarchidae* family, the three-Line Grunt (*Parapristipoma trilineatum),* and the Blue-Eyed Plecostomus (*Plecostomus spp).*

Salmonidae Family

TAXON NAME	COMMON NAME
Coregonus clupeaformis	Lake whitefish
Coregonus hoyi	Bloater
Oncorhynchus keta	Chum salmon
Oncorhynchus gorbuscha	Pink salmon
Oncorhynchus kisutch	Coho salmon (silver salmon)
Oncorhynchus masou	cherry salmon (masou salmon)
Oncorhynchus nerka	Sockeye salmon

Oncorhynchus tshawytscha	(chinook salmon)
Prosopium cylindraceum	Round whitefish
Oncorhynchus clarki	Cutthroat trout
Oncorhynchus mykiss	Rainbow trout
Salmo salar	Atlantic salmon
Salmo trutta	Brown trout
Salmo trutta X S. fontinalis	Tiger hybrid-trout
Salvelinus alpinus	Arctic charr
Salvelinus confluentus	Bull trout
Salvelinus fontinalis	Brook trout
Salvelinus leucomaenis	Japanese charr (white spotted charr)
Salvelinus malma	Dolly varden (Miyabe charr)
Salvelinus namaycush	Lake trout
Thymallus thymallus	Grayling

Some Members of the *Serranidae* Family

TAXON NAME	COMMON NAME
Centropristis ocyurus	Bank sea bass
Centropristis philadelphicus	Rock sea bass
Centropristis striata	Black sea bass
Diplectrum bivittatum	Dwarf sandperch
Diplectrum formosum	Sand perch
Epinephelus flavolimbatus	Yellowedge grouper
Epinephelus morio	Red grouper
Serranus phoebe	Tattler
Serranus tortugarum	Chalk bass

Some Members of the *Sparidae* family

TAXON NAME	COMMON NAME
Archosargus probatocephalus	Sheepshead
Archosargus rhomboidalis	Sea bream
Calamus penna	Sheepshead porgy
Lagodon rhomboides	Pinfish
Pagrus Major	Red Sea bream
Sparus aurata	Gilthead Sea bream
Stenotomus chrysops	Scup

Some Members of the *Cichlidae* family

TAXON NAME	COMMON NAME
Aequidens latifrons	Blue acara
Cichlisoma nigrofasciatum	Congo cichlid
Crenichichla sp.	Pike cichlid
Pterophyllum scalare	Angel fish
Tilapia mossambica	Mozambique mouth breeder
Oreochromis spp	Tilapia
Sarotherodon aurea	Golden Tilapia

Some Members of the *Centrarchidae* family

TAXON NAME	COMMON NAME
Ambloplites rupestris	Rock bass
Centrarchus macropterus	Flier
Elassoma evergladei	Everglades pigmy sunfish
Elassoma okefenokee	Okefenokee pigmy sunfish
Elassoma zonatum	Banded pigmy sunfish
Enneacanthus gloriosus	Bluespotted sunfish
Enneacanthus obesus	Banded sunfish
Lepomis auritus	Redbreast sunfish
Lepomis cyanellus	Green sunfish
Lepomis cyanellus X L. gibbosus	Green x pumpkinseed
Lepomis gibbosus	Pumpkinseed
Lepomis gulosus	Warmouth
Lepomis humilis	Orange-spotted sunfish
Lepomis macrochirus	Bluegill
Lepomis megalotis	Longear sunfish
Micropterus coosae	Shoal bass
Micropterus dolomieui	Smallmouth bass
Micropterus punctulatus	Spotted bass
Micropterus salmoides	Largemouth bass
Pomoxis annularis	White crappie
Pomoxis nigromaculatus	Black crappie

In a further embodiment, the animal is a companion animal or a human. For purposes of the present invention, the term "companion" animal shall be understood to include all animals – horses (equine), cats (feline), dogs (canine), and rodents, including mice, rats, guinea pigs, rabbit species, and avians, such as pigeons, parrots, and the like.

Birds receiving such vaccination can be associated with either commercial or noncommercial aviculture. These include *e.g.*, *Anatidae*, such as swans, geese, and ducks, *Columbidae*, *e.g.*, doves and pigeons, such as domestic pigeons, *Phasianidae*, *e.g.*, partridge, grouse and turkeys, *Thesienidae*, e.g, domestic chickens, *Psittacines*, *e.g.*, parakeets, macaws, and parrots, *e.g.*, raised for the pet or collector market. Chickens are exemplified hereinbelow.

Sources of Wild-Type Isolates

Generally, the attenuated *C. perfringens* organisms of the invention can be produced starting with a wild-type *C. perfringens* that has originally been isolated from any infected animal of interest, as discussed *supra*, and/or from the environment. The environment includes any material that contains viable *C. perfringens* organisms and/or

viable *C. perfringens* spores including, for example, contaminated food, soil, water, animal bedding material, feces, and the like.

Justin *et al.*, [*Biochemistry 41*, 6253-6262 (2002)] characterized *alpha*-toxins from different strains of *C. perfringens* that are almost identical in sequence and biochemical properties. However, Justin *et al.*, also describe a strain that was isolated from an avian source (a swan) that had an *alpha*-toxin exhibiting a large degree of sequence variation and altered substrate specificity compared to the other strains. For this reason, it is believed that most isolates will, when converted to an attenuated form, elicit protective immunity against the *alpha*-toxin of many other naturally occurring strains of *C. perfringens*. Nevertheless, given the possibility of *alpha*-toxin variation between isolates, it will often be advantageous to isolate and attenuate *C. perfringens* organisms from the animal species for which an anti-*C. perfringens* vaccine is desired. The isolate exemplified hereinbelow was isolated from chicken, and tested in that species.

Vaccines

The attenuated *C. perfringens* organisms of the invention are generally formulated into pharmaceutically acceptable vaccine compositions. The vaccine compositions are formulated according to the route of administration and are compatible with the active antigenic agent. The active antigenic agent is, for example, one or more strains of attenuated *C. perfringens* type A organisms according to the invention. Optionally, the vaccine composition can also include one or more types of non-toxic *alpha*-protein, in combination with the attenuated *C. perfringens* type A organisms.

For example, substantially all of the attenuated *C. perfringens* type A organisms included in the vaccine composition are alive and viable, although for certain specific situations, *e.g.*, for immunizing certain humans or immune-compromised animals, the vaccine will exclusively comprise killed attenuated *C. perfringens* type A organisms.

The vaccine composition includes physiologically compatible buffers and/or salts, in optional combination with adjuvants and/or optional immune enhancers or stimulants (co-administered or administered in series, *e.g.*, before or after vaccination).

Suitable immune stimulants include, but are not limited to, cytokines, growth factors, chemokines, supernatants from cell cultures of lymphocytes, monocytes, cells from lymphoid organs, cell preparations or cell extracts (*e.g.*

Staphylococcus aureus or lipopolysaccharide preparations), mitogens, or adjuvants including low molecular weight pharmaceuticals. An immune stimulant can be administered *in ovo* at any time during incubation. In a particular aspect, the immune stimulant is administered in the medium containing the attenuated *C. perfringens* type A organisms.

Methods of Administering Vaccine

The above-described inventive vaccines are administered, for example, by injection or inoculation by one or a combination of the following routes: oral, intranasal, parenteral, subcutaneous, scarification, and/on intramuscular administration in any suitable, art-known formulation, *e.g.*, a compatible buffer and/or a physiologically acceptable saline, in optional combination with adjuvants and/or immune enhancers or stimulants (co-administered or administered in series, *e.g.*, before or after vaccination). For orally-administered vaccines/vaccination methods, any of the physiologically-suitable buffers or suspending agents that are known to the art are readily employed. In addition, the composition can be incorporated, *e.g.*, admixed into drinking water or sprayed onto food pellets, dusted or sprayed onto corn or other grains, and the like.

The gastrointestinal tract is a common site of *C. perfringens* infection, and therefore oral administration is contemplated as one method of inoculation. The presence of the gastrointestinal tract by the live attenuated *C. perfringens* organisms of the invention is contemplated to elicit localized protective immune reactions in the mucosal layer of the gastrointestinal tract, and may also act competitively to prevent subsequent colonization by wild-type *C. perfringens.*

For avians, such as domestic fowl, including chickens, ducks, geese, etc., the oral method, or injection *in ovo* are among the useful routes for vaccination. The *in ovo* route is exemplified hereinbelow, and produced active immunization and protection from challenge in the hatched chickens.

Foreign Gene Expression by *C. Perfringens*

Using the techniques developed in the preceding examples, any gene not naturally occurring, *i.e.*, foreign to *C. Perfringens* optionally can be inserted into the chromosomal DNA of *C. perfringens*. For expression of foreign proteins, gene fusions can be made which preserve the sequences flanking the *alpha*-toxin gene, the *alpha*-toxin promoter and its signal sequence. In one embodiment, most of the coding

sequence of the *plc* gene is replaced with the foreign gene. The remaining nucleotides of the *plc* gene downstream of the inserted gene are out of frame, and therefore no functional *alpha*-toxin is produced. Alternatively, the foreign gene can be inserted in-frame to the nucleotide sequence encoding the *alpha*-toxin mutein forming an *alpha*-toxin - foreign protein fusion protein.

Oligonucleotide primers for the foreign gene can be synthesized, *e.g.*, with an N-terminal FLAG tag sequence and appropriate restriction sites. The PCR products can be cloned into the suicide vector; the FLAG tag and foreign gene are inserted in frame with the *alpha*-toxin signal sequence. The foreign protein is expressed under control of the *alpha*-toxin promoter and targeted for secretion by the *plc* signal sequence. The secreted foreign protein can be detected in the supernatant media by Western blot using an anti-FLAG antibody.

Any suitable foreign gene may be inserted into the *C. perfringens* genome in this manner. These include, for example, DNA encoding antigens from pathogens of the gastrointestinal tract, including, *e.g.*, antigenic proteins of bacteria such as *E. coli*, salmonella species, campylobacter species, lawsonia species, and the like; antigenic proteins of parasites such as eimeria species, isospora species, cryptosporidium species and the like; and antigenic proteins of viruses such as rotaviruses, coronaviruses, and the like, in order to immunize the animal treated with such a recombinant *C. perfringens*. Other proteins that may be expressed by such recombinant *C. perfringens* include therapeutic proteins or peptides. Optionally, these include peptides that are endogenous to the gastrointestinal tract, including trefoil factor, or any type of art-known cytokine, *e.g.* chicken IL-18, and the like.

One such *C. perfringens* construct expresses the chicken IL-18 protein. Administration of live bacteria containing the gene fusion will allow delivery of therapeutic doses of IL-18 into the gut yet be relatively innocuous to the host animal by virtue of the absence of *alpha*-toxin production. Other therapeutic agents may also be expressed using this system.

Numerous references are cited herein, the content of each of which is incorporated by reference herein in its entirety. The following specific examples are included for purposes of illustration, and are not intended to limit the scope of the invention, unless otherwise indicated.

EXAMPLE 1
C. PERFRINGENS ALPHA-TOXIN DELETANT HOMOLOGY VECTOR

A homology plasmid vector 1162-55-20 useful in the construction of *C. perfringens alpha*-toxin deletants was created. The plasmid incorporates several important elements; the replication region from the *E. coli* plasmid pUC18; the *C. perfringens* chloramphenicol (*catP*) and erythromycin (*ermBP*) resistance genes (both of which are also expressed in *E.coli*); and a *C. perfringens alpha*-toxin gene (*plc*) inactivated by a specific deletion of 9 amino acids. Plasmid 1162-55-20 was created in several steps, as follows.

First the *plc* gene was cloned from a recent avian isolate of *C. perfringens* (strain CP6). The sequence of *C. perfringens* strain 13 (Genbank NC 003366; SEQ ID NO: 2) was used to design oligonucleotide primers to be used in the cloning of the *plc* gene. These and all subsequent primers were obtained commercially form Sigma Genosys, Woodlands, TX. The upstream primer located within the *yplc* gene (CPE0035), 5' AGCTGCATAAGCAAAAGTTCCAACTC 3' (SEQ ID NO: 14) corresponds to nucleotides 47675-47700 of strain 13 (SEQ ID NO: 2). The downstream primer located within the *cobW* gene (CPE0037), 5' GCAGAAACTCTTCTTAGACCTATTCTTTTAGGC 3' (SEQ ID NO: 15), is complementary to nucleotides 50597-50629 of strain 13. These primers were used along with genomic DNA from *C. perfringens* strain CP6 in a long range polymerase chain reaction (PCR). A product of 2955 base pairs from the upstream *yplc* gene through the downstream *cob W* gene was predicted from the known sequence of strain 13 (as illustrated by FIG. 1). The *alpha*-toxin promoter, signal sequence and *plc* gene (CPE0036) coding sequence are contained within this fragment, *i.e.* between the upstream *yplc* gene and the downstream *cob W* gene. The PCR 2955 base pair fragment was then cloned into cloning vector pCR-Blunt (Invitrogen Corporation, Carlsbad, CA) resulting in plasmid 1162-52.1. The nucleotide sequence of the *plc* coding region of the 2955 base pair fragment of strain CP 6, was determined from this fragment (SEQ ID NO: 22; and corresponding polypeptide is SEQ ID NO: 23) and was shown to be substantially homologous as that of the *plc* gene of *C. perfringens* strain 13 (SEQ ID NO: 2).

Next the *E. coli* replication region and the *C. perfringens* resistance genes were cloned from shuttle plasmid pJIR418 (Sloan, *et al*, 1992, *Plasmid 27,* 207-219; Genebank M77169). Plasmid pJIR418 was digested with restriction enzymes Bam HI and Spe I and the ends filled in with Klenow polymerase. Ligation of the large fragment produced plasmid 1162-45.1 and restored the Bam HI restriction site. This plasmid

retains the *E. coli* replication region, but unlike pJIR418, it does not contain a *C. perfringens* origin of replication. The plasmid is therefore capable of autonomous replication in *E. coli*, but not in *C. perfringens*.

In the next step the C-terminal half of the *plc* gene was sub-cloned into the intermediate plasmid 1162-45.1. Digestion of plasmid 1162-52.1 with Bam HI and Eco RI released a 1742 base pair fragment containing the C-terminal portion of the *alpha*-toxin gene from the unique Bam HI site located with in the *plc* gene downstream through the *cob W* gene to an Eco RI site located in the multiple cloning site of the parent plasmid. The 1742 base pair fragment was cloned between the Bam HI and Eco RI sites located within the multiple cloning site of plasmid 1162-45.1. In the resulting plasmid 1162-53.7, the C-terminal half of the *plc* gene is in the same transcriptional orientation as the *catP* and *ermBP* genes of the parental plasmid.

In the final step the N-terminal half of the *plc* gene was cloned into the unique Bam HI site of plasmid 1162-53.7. This was accomplished by creating a PCR fragment derived from the *alpha*-toxin gene sub-cloned in plasmid 1162-52.1. The upstream primer, 5' ggatccAGCTGCATAAGCAAAAGTTCCAACTC 3' (SEQ ID NO: 16) was identical to the previously noted *yplc* primer (SEQ ID NO: 4) except that a flanking Bam HI site (lowercase) was included. The downstream primer located within the *alpha*-toxin gene, 5' ggatccaATGCATTCTTATCATAATCTGGATAAGTAGAACC 3' (SEQ ID NO: 17) was complementary to nucleotides 48824-48857 of strain 13 and included a flanking Bam HI restriction site and a spacer nucleotide to maintain the reading frame (lowercase). The Bam HI fragment resulting from PCR with these primers and plasmid 1162-52-1 template DNA was cloned into the unique Bam HI site of plasmid 1162-53.7. A plasmid containing the two *plc* gene regions in the same transcriptional orientation was isolated. This plasmid 1162-55.20 contains the *plc* gene with the desired nine amino acid deletion and the addition of a leu residue between the ala 61 and asp 71 of the wild-type toxin (see FIG. 2). DNA sequencing of the plasmid confirmed the reading frame and the net deletion of 24 codons (encoding eight amino acid residues).

EXAMPLE 2
CONSTRUCTION OF *CLOSTRIDIUM PERFRINGENS* RECOMBINANT CPERF001

The deleted version of the *plc* gene constructed in Example 1 was introduced into *C. perfringens* using the following strategy. The homology vector 1162-55.20 is termed a *C. perfringens* "suicide plasmid" because its *C. perfringens* origin of replication has been removed.

When this plasmid was transformed into *C. perfringens* it is unable to replicate and does not survive. However, if the transformed bacteria are placed under chormaphenicol and/or erythromycin selection, the plasmid DNA can be forced to recombine into the bacterial genome via homology to the *plc* gene. The resulting recombinant bacteria is termed an integrant. The integrant contained a copy of the introduced homology vector that was integrated at the *plc* gene locus. Thus, the resulting integrant contained two copies of the *plc* gene, the original normal copy, and the introduced deleted version. The introduced resistance genes were located between the two copies of the *plc* gene. When antibiotic selection was removed from the integrant, recombination occurred between the two copies of the *plc* gene. This recombination event can produce one of two outcomes. In both cases, the DNA intervening between the two copies of the *plc* gene (including the resistance genes) has been removed. In the first outcome, the normal *plc* gene was restored, resulting in recovery of the original parent strain. In the second outcome, the normal *plc* gene is replaced by the deleted copy, producing the desired *alpha*-toxin deletant construct. Since the *alpha*-toxin of the deletant strain is inactivated, this strain was non-hemolytic. Therefore the desired deletant integrant was identified by screening for non-hemolytic clones on blood agar plates.

Because the recombination event resulting in the desired integrant was expected to occur at a low frequency, it was critical to employ parent *C. perfringens* strains having high transformation efficiency. Therefore several recent avian isolates of *C. perfringens* were analyzed for transformation efficiency. The isolates were transformed with the shuttle plasmid pJIR418 as described by Allen and Blaschek (*Applied and Environmental Microbiology 54*:2322 (1988)) with slight modifications (described below). Strain 1240 exhibited the highest transformation efficiency (see Table 1), 9.2 $x10^6$ transformants/μg of pJIR418 plasmid DNA. This strain was chosen to be the parent strain for construction of deletant CPERF001. Strain 29 was chosen to be the parent strain for CPERF002.

Table 1
Transformation Efficiency of *C. perfringens* Avian Isolates

C. perf. A Strain	Transformants/μg of pJIR418
29	3.6×10^4
23	5.6×10^2
1220	4.0×10^6
1240	9.2×10^6
CP-2	None

1230	7.1×10^3
5227	None
5230	None

Source of above listed wild-type strains was Dr. J. Glenn Songer,
Dept. of Veterinary Sciences and Microbiology,
University of Arizona, Tucson, Arizona 85721

C. perfringens strain 1240 cells from an overnight anaerobic culture in TSYC media (30 g/l tryptic soy broth, 5 g/l yeast extract, 0.5 g/l cysteine) were diluted 1:25 and grown to A_{600}=0.5436. After centrifugation of 100 ml of cells at 18,000 x g for 10 minutes, electrocompetent cells were prepared by twice resuspending cells in an equal volume of pre-reduced sucrose magnesium phosphate ("SMP" was prepared as 270 mM sucrose, 1 mM $MgCl_2$, 7 mM $NaPO_4$, pH 7.3) buffer followed by a final resuspension with 0.5 ml SMP. This resulted in a final volume of ~2.0 ml.

Aliquots of 100 µl of cells were electroporated with 4 µg of plasmid 1162-55.20 in 0.2 cm cuvettes. A Bio-Rad Gene Pulser II was used at 1.37 kilovolts, 100 Ohms resistance and 50 microfarads capacitance. Immediately after electroporation, cells were diluted with 2.0 ml of pre-reduced tryptic soy yeast cysteine media ("TSYC" was prepared as 30 g tryptic soy broth, 5g yeast extract, 0.5 g cysteine, 950 ml water) and incubated for three hours at 37°C in an anaerobic jar. After the recovery period, dilutions of cells were plated on TSYC + 25 µg/ml of chloramphenicol plates. These plates were incubated at 37°C overnight in an anaerobic jar.

After overnight growth, an average of 50 chloramphenicol-resistant colonies per microgram of 1162-55.20 DNA was observed. Single colonies of seven putative integrants were grown up in nonselective TSYC media for four successive grow outs and plated onto nonselective blood agar plates. One of the non-selected stocks, 1192-31.7, exhibited several non-hemolytic colonies. Twenty-one of these non-hemolytic colonies were patched to nonselective master plates and replica plated on TSYC + chloramphenicol plates. Two of the 21 colonies were chloramphenicol sensitive.

One of the chloramphenicol-sensitive putative deletants, 1192-32.14, was grown up along with the 1240 wild-type and the integrant 1192-31.7 and plated on blood agar plates. The 1240 wild-type strain showed clear zones of beta hemolysis whereas the 1192-32.14 deletant was a pure culture of non-hemolytic colonies and was renamed CPERF001.

Other blood plates were used as masters and replica plated to nonselective and selective media. The 1240 wild-type and CPERF001 were chloramphenicol and

erythromycin sensitive. The integrant, 1192-31.7, was both chloramphenicol and erythromycin resistant as expected.

To rule out the possibility that the antibiotic resistance genes were present but not expressed in CPERF001, PCR primers specific for the chloramphenicol and erythromycin genes were synthesized for use in PCR reactions. Genomic DNAs were prepared from the wild-type, integrant and CPERF001 strains and used as templates for PCR reactions with the antibiotic gene primers. Results of the PCR analysis showed positive response only from the suicide plasmid and the integrant and not the parent 1240 or deletant CPERF001. This confirms the predicted loss of the resistance gene sequences.

To confirm the deletion within the *alpha*-toxin gene, a 1086 bp region surrounding the deletion was amplified by PCR using appropriate *alpha*-toxin specific primers. The amplified fragment was cloned and sequenced. Sequencing results confirmed the deletion of the nine amino acids from tyr 62 through trp 70 of the *alpha*-toxin gene and the insertion of a single leu (see FIGs. 2A-2C).

CPERF001 was assayed for expression of the inactivated *alpha* toxoid protein. After 6 hours of anaerobic growth, aliquots of 1ml of cells were collected and centrifuged. Fifteen microliters of the unconcentrated supernatant media were analyzed by polyacrylamide gel electrophoresis and subjected to Western Blot analysis with a rabbit polyclonal antibody directed against recombinant *alpha*-toxin protein (*Vaccine* 11(12): 1253-1258 (1993)). The results showed specific antibody reactivity with a protein of the expected size for the *alpha* toxoid protein.

CPERF001 is a genetically engineered deletant strain of *C. perfringens* Type A. This strain secretes an inactivated toxoid form of the *C. perfringens alpha*-toxin. Because this strain no longer expresses active *alpha*-toxin yet retains a significant portion of the toxin's antigenicity, it will be useful as a vaccine to protect against disease caused by *C. perfringens*.

EXAMPLE 3
CONSTRUCTION OF *CLOSTRIDIUM PERFRINGENS* RECOMBINANT CPERF002

In order to compensate for the lower transformation efficiency of *C. perfringens* strain 29 (see Table 1, *supra*) a new homology vector was constructed. The new vector incorporated *C. perfringens* sequences cloned directly from the strain 29 genome. This

was predicted to result in a more efficient recombination step. The new vector was 1192-38.3 created as follows.

In the first step the *E. coli* replication region and the *C. perfringens* resistance genes were cloned from shuttle plasmid pJIR418 (Sloan et al, *Plasmid 27*, 207 (1992); Genebank M77169). Plasmid pJIR418 was digested with restriction enzyme NdeI. Ligation of the large fragment produced plasmid 1192-23.1. This plasmid lacks a *C. perfringens* origin of replication, but unlike the plasmid 1162-45.1 that was constructed in Example 1, it retains the entire multiple cloning site of pJIR418

In the next step the C-terminal half of the *plc* gene was sub-cloned into the intermediate plasmid 1192-23.1. Genomic DNA from strain 29 was used as a template for long range PCR. The region of the *plc* gene (*alpha*-toxin) from the Bam HI site through a portion of the CPE0038 gene was amplified. The upstream *plc* primer, 5' CTGGGATCCTGATACAGATAATAATTTCTCAAAGGAT 3' (SEQ ID NO: 18) corresponds to nucleotides 48880-48916 of strain 13 (Genbank NC 003366). The downstream primer within the CPE0038 gene, 5' actctgcagTTGTCATATCAATTAAATTAACTATAATCCC 3' (SEQ ID NO: 19) is complementary to nucleotides 51244-51275 of strain 13 and contains flanking nucleotides including a Pst I restriction site (lowercase). A product of 2402 base pairs was obtained and digested with restriction enzymes Bam HI and Pst I. This fragment was ligated with the large fragment of Bam HI and Pst I digested 1192-23.1 to produce plasmid 1192-36.10.

In the final step the N-terminal half of the *plc* gene was cloned via PCR. The upstream primer, 5' actgagctcCTAGACACTTTGCTTCAATATTTGGGAA 3' (SEQ ID NO: 20) corresponds to nucleotides 46513-46540 of strain 13 and includes flanking nucleotides and a Sac I site (lowercase). The downstream primer, 5' actggatccGCATTCTTATCATAATCTGGATAAGTAGAACC 3' (SEQ ID NO: 21) is complementary to nucleotides 48824-48855 of strain 13 and includes flanking nucleotides and a Bam HI site. A 2363 base pair product was produced and this was digested with Sac I and Bam HI restriction enzymes. The digested fragment was ligated with the large fragment of Sac I and Bam HI digested 1192-36.10 to produce plasmid 1192-38.3. Subsequent sequencing of the region flanking the *plc* Bam HI site in 1192-38.3 confirmed the deletion of nine amino acids from tyr 62 through trp 70.

Plasmid 1192-38.3 was used to electroporate *C. perfringens* strain 29 cells. Strain 29 was previously shown to transform with an efficiency of 3.6 x104

transformants/μg of pJIR418 plasmid DNA. Strain 29 cells were grown and electroporated as in Example 2 with the exception that a liquid selection technique was used after the three hour recovery period. Instead of plating, 0.67 ml aliquots of cells were diluted into 12 ml of TSYC + 25 μg/ml chloramphenicol and grown overnight at 37°C in an anaerobic jar. This modification was used because of the lower transformation and plating efficiencies of strain 29 versus 1240. After overnight growth, cells were diluted again in selection media. Cells from the second grow out were then passed five times without selection prior to plating on blood agar plates. None of the colonies from the blood agar plates were non-hemolytic. Two blood plates were then replica plated to TSYC, TSYC + 25 μg/ml chloramphenicol and TSYC + 50 μg erythromycin plates. All colonies were erythromycin sensitive but only one of 130 colonies was chloramphenicol sensitive. This colony, 1192-45.4B, was renamed CPERF002. PCR analysis of genomic DNA from CPERF002 with *alpha*-toxin specific primers showed a positive band for *alpha*-toxin. This band was smaller than the corresponding band from wild-type strain 29 DNA. Primers specific for the chloramphenicol and erythromycin resistance genes failed to amplify CPERF002 DNA but showed strong positive bands from plasmid 1192-38.3. Sequencing of the CPERF002 *alpha*-toxin confirmed the nine amino acid deletion (see FIG. 3).

CPERF002 was assayed for expression of the inactivated *alpha* toxoid protein. After 6 hours of anaerobic growth, aliquots of 1ml of cells were collected and centrifuged. Fifteen microliters of the unconcentrated supernatant media were analyzed by polyacrylamide gel electrophoresis and subjected to Western Blot analysis with a rabbit polyclonal antibody directed against recombinant *alpha*-toxin protein (*Vaccine 11*: 1253 (1993)). The results showed specific antibody reactivity with a protein of the expected size for the *alpha* toxoid protein.

CPERF002 is a genetically engineered deletant strain of *C. perfringens* Type A. This strain secretes an inactivated toxoid form of the *C. perfringens alpha*-toxin. Because this strain no longer expresses active *alpha*-toxin yet retains a significant portion of the toxin's antigenicity, it will be useful as a vaccine to protect against disease caused by *C. perfringens*.

EXAMPLE 4
C. PERFRINGENS DELETANT VACCINES

The CPERF001 and CPERF002 deletant vaccine strains described in Examples 2 and 3 were evaluated for their ability to provide protection against challenge with wild-type *C. perfirngens*. The first part of the study was designed to determine if the administration of the live vaccine strains had any adverse effect on the hatchability of embryonated eggs. The assignment of experimental groups and the safety results are described by Tables 2

TABLE 2

SAFETY RESULTS

Group*	Vaccine (dose)**	Number of Eggs Hatched (%)
1	CPERF001 (0.8×10^2)^x	18 (90%)
2	CPERF001 (0.8×10^3)	17(85%)
3	CPERF001 (0.8×10^4)	11 (55%)
4	CPERF001 (0.8×10^5)	16 (80%)
5	CPERF002 (1.9×10^2)	16 (80%)
6	CPERF002 (1.9×10^3)	17 (85%)
7	CPERF002 (1.9×10^4)	14 (70%)
8	CPERF002 (1.9×10^5)	12 (60%)
9	Strain 1240 (1.5×10^3)	16 (80%)
10	Strain 29 (3.7×10^3)	13 (65%)
11	Media Controls	19 (95%)
12	Uninoculated Controls	18 (90%)

* 20 eggs per group
** 100 µl dose given *in ovo* at 18 days of embryonation (IM)
^{x} Titer per dose is measured as colony forming units (cfu")

At a dose of 10^3 or less (groups 1,2, 5, and 6), hatchability in these groups was not significantly lower than in the group inoculated with media only (group 11) or the group that was not inoculated (group 12). At the lowest dose CPERF001 showed the same hatchability as the media control and better than the uninoculated control group.

Because the higher doses of the deletants may have had an adverse effect on the hatchability of the eggs, only the two lowest dosage groups for each deletant were included in the efficacy portion of the study. These groups, along with the media control birds (group 11), were challenged with (wild-type) *C. perfringens* strain CP6 that was administered orally at approximately 10^8 cfu/ml/bird when the birds were 20, 21 and 22 days of age. All birds were necropsied at 25 days of age and lesions within the small intestine were scored using the rating scale for necrotic enteritis summarized

below.

0 Score	Necrotic Enteritis 1+	Necrotic Enteritis 2+	Necrotic Enteritis 3+	Necrotic Enteritis 4+
No NE gross lesions on small intestine; intestine has normal elasticity (rolls back on itself after being opened).	Thin and flaccid intestinal wall (intestine remains flat when opened and doesn't roll back into normal position); excess or thickened mucus covering mucus membrane or focal or multifocal mild reddening of the mucosa or congestion of the serosal vessels.	Single or few multifocal areas of reddening and swelling of the intestinal wall; single or few multifocal areas of ulceration of necrosis of the intestinal mucosa.	Extensive multifocal areas of necrosis and ulceration of the intestinal mucosa ± significant hemorrhage or layer of fibrin or necrotic debris on the mucosal surface (Turkish towel appearance).	Dead animal with NE gross lesions scored 2+ or above.

Scoring with minor modifcations is according to Charles Hofacre, D.V.M., M.A.M., Ph.D., University of Georgia, Poultry Diagnostic and Research Center, 953 College Station Road, Athens, GA 30602,

Five (5) birds from the unvaccinated control group (not challenged) were necropsied at the end of the study to confirm there was no exposure to *C. perfringens* during the course of the study. The results are presented in Table 3.

TABLE 3

TREATMENT GROUPS FOR PROTECTION* RESULTS

Group*	Vaccine	*In-ovo* Vaccine Dose (cfu)	Days of age at Challenge with *C. perfringens*	N	Mean
1	CPERF001	$1x10^2$	20,21 and 22	9	0.67
2	CPERF001	$1x10^3$	20,21 and 22	13	0.46
5	CPERF002	$1x10^2$	20,21 and 22	12	1.33
6	CPERF002	$1x10^3$	20,21 and 22	12	1.08

11	Media control	None	20,21 and 22	15	1.80
12	None	None	Not challenged	5	0.00

* necropsy at 25 days of age

Scores for Groups 1 and 2 were each statistically significant lower compared to Groups 11 (Wilcoxon Exact Rank Sum Test $p \leq 0.0250$). Mean scores for Groups 5 and 6 were lower than Group 11 but not statistically different (Wilcoxon Exact Rank Sum Test $p \geq 0.2177$). An estimate of vaccine efficacy was performed according to the procedure described by David Siev [*Journal of Modern Applied Statistical Methods* Vol. 4, No. 2, 500-508 (2005)]. Vaccine efficacy in reducing disease severity was estimated at 54% for Group 1, 65% for Group 2, 20% for Group 5 and 27% for Group 6 when compared to Group 11.

EXAMPLE 5
CONSTRUCTION OF A *C. PERFRINGENS* SWINE DELETANT

The strategies used in Examples 1 and 2, *supra*, are used to construct *alpha*-toxin deletion mutants from a swine strains of *C. perfringens*. Initially, field isolates from diseased swine are electroporated with plasmid pJIR418 to assess their transformability. Isolates yielding $\geq 10^4$ transformants per microgram of plasmid DNA and susceptible to either chloramphenicol or erythromycin are candidates for deletion.

Genomic DNA from the candidate strains is used as a template for long range PCR of the *plc* (*alpha*-toxin) gene and flanking sequences. After subcloning of the PCR products, the *alpha*-toxin gene and flanking regions are sequenced and restriction mapped. New oligonucleotide primers are synthesized with flanking restriction sites and the products of two separate amplifications are cloned into the suicide plasmid 1192-23.1 to create the 27 base pair deletion as in Example 1.

The swine suicide vector is electroporated into the corresponding swine strain of *C. perfringens* and deletion mutants are isolated using the methods described in Example 2, *supra*. Deletion mutants are confirmed by the absence of beta hemolysis on blood agar plates and by DNA sequencing of the *alpha*-toxin gene.

This construct is a genetically engineered deletant strain of *C. perfringens* Type A. This strain secretes an inactivated toxoid form of the *C. perfringens alpha*-toxin. Because this strain no longer expresses active *alpha*-toxin yet retains a significant portion of the toxin's antigenicity, it is useful as a vaccine to protect swine against

disease caused by *C. perfringens*.

BIOLOGICAL DEPOSIT

Cultures of the following biological materials have been deposited with the following international depository:

American Type Culture Collection (ATCC) 10801 University Boulevard, Manassas, Va. 20110-2209, U.S.A., under conditions that satisfy the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

Organism	Accession No.	Date of Deposit
Clostridium perfringens CPERF/ΔαToxin 365-054	PTA7364	February 7, 2006
Clostridium perfringens CPERF/ΔαToxin 365-053	PTA7365	February 7, 2006

WE CLAIM:

1. A nucleic acid molecule that encodes a substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin; wherein the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3 minus at least 9 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

2. The nucleic acid molecule of claim 1 wherein the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3 minus at least 12 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

3. The nucleic acid molecule of claim 2 wherein the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3 minus at least 18 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

4. The nucleic acid molecule of claim 1 wherein the mutein *alpha*-toxin comprises SEQ ID NO: 3 minus 9 consecutive amino acid residues ranging from Tyr_{62} through Trp_{70}.

5. The nucleic acid molecule of claim 1 comprising the nucleic acid sequence of SEQ ID NO: 2; wherein nucleotides 268-294 of the nucleic acid molecule are deleted.

6. The nucleic acid molecule of claim 5 wherein the deleted nucleotides are replaced by a nucleotide sequence encoding a single Leu residue.

7. A substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin; wherein the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3 minus at least 9 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

8. The substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of claim 7 wherein the mutein *alpha*-toxin comprises the amino acid sequence of SEQ ID NO: 3

minus at least 12 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

9. The substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of claim 8, wherein said mutein comprises the amino acid sequence of SEQ ID NO: 3 minus at least 18 consecutive amino acid residues; and wherein one of the deleted amino acid residues is His_{68}.

10. The substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of claim 7, wherein nine consecutive amino acid residues that range from Tyr_{62} through Trp_{70}, are deleted and replaced by a single Leu residue.

11. An attenuated *Clostridium perfringens* organism constructed by integrating the nucleic acid molecule of claim 1 into a chromosome of a non-attenuated *Clostridium perfringens* organism.

12. The attenuated *Clostridium perfringens* organism of claim 11 that is substantially nontoxic.

13. The attenuated *Clostridium perfringens* organism of claim 11 wherein the nucleic acid molecule is located at a position on the chromosome that is homologous to the location of a nucleic acid molecule encoding a wild-type *alpha*-toxin present in the non-attenuated *Clostridium perfringens*.

14. The attenuated *Clostridium perfringens* organism of claim 11 that is a type A *Clostridium perfringens*.

15. The attenuated *Clostridium perfringens* organism of claim 11 wherein the non-attenuated *Clostridium perfringens* from which it was constructed was isolated from a host animal that is either a mammal or an avian.

16. The attenuated *Clostridium perfringens* organism of claim 15, wherein said mammal is selected from the group consisting of bovine, ovine, and porcine.

17. The attenuated *Clostridium perfringens* organism of claim 15 wherein said avian is a chicken, a turkey, a goose, a duck, a swan, a dove, a pigeon, a grouse, or a partridge.

18. A vaccine comprising the attenuated *Clostridium perfringens* organism of claim 11.

19. The vaccine of claim 18 further comprising one or more of the following: a pharmacologically acceptable buffer, excipient, or an adjuvant.

20. A method of inducing immunity to *Clostridium perfringens* in an animal, comprising administering to the animal an immunologically effective dose of the vaccine of claim 18.

21. The method of claim 20 wherein the vaccine is administered by one or more of the following routes: oral, intramuscular, intravenous, intradermal, subcutaneous, or intranasal.

22. The method of claim 20 wherein the vaccine is top-dressed on the feed of the animal.

23. The method of claim 20 wherein the vaccine is sprayed onto the animals to provide for oral administration.

24. An animal feed that comprises the vaccine of claim 18.

25. The attenuated *Clostridium perfringens* organism of claim 11 that comprises at least one gene encoding a non-*Clostridium perfringens* polypeptide.

26. The attenuated *Clostridium perfringens* organism of claim 25 wherein said non-*Clostridium perfringens* polypeptide is a non-bacterial polypeptide.

27. The attenuated *Clostridium perfringens* organism of claim 26 wherein the non-bacterial polypeptide is an avian or mammalian polypeptide.

28. The attenuated *Clostridium perfringens* organism of claim 27 wherein the non-bacterial polypeptide is an avian polypeptide.

29. The attenuated *Clostridium perfringens* organism of claim 27 wherein the non-bacterial polypeptide is a cytokine.

30. The attenuated *Clostridium perfringens* organism of claim 29 wherein the cytokine is chicken IL-18.

31. An antibody that selectively binds to an epitope found in *Clostridium perfringens alpha*-toxin but missing from the substantially nontoxic mutein of *Clostridium perfringens alpha*-toxin of claim 11 wherein said antibody can distinguish said substantially nontoxic mutein from a wild type *Clostridium perfringens alpha*-toxin.

32. A test kit for identifying whether a subject animal has been naturally infected by a *Clostridium perfringens* organism comprising the antibody of claim 31.

33. A method of identifying an animal that has been naturally infected by a *Clostridium perfringens* organism from one vaccinated with an attenuated *Clostridium perfringens* organism, said method comprising:
(a) contacting a fluid sample from the animal with the antibody of claim 32,
(b) determining whether the antibody reacts with the fluid sample;
wherein when the antibody reacts with the fluid sample, the animal is identified as one that has been naturally infected by a *Clostridium perfringens* organism.

34. A *Clostridium perfringens* organism that is CPERF/ΔαToxin 365-054 having ATCC Deposit No. PTA7364.

35. A *Clostridium perfringens* organism that is CPERF/ΔαToxin 365-053 having ATCC Deposit No. PTA7365.