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(72) Inventeurs/Inventors:
TITBALL, RICHARD W., GB;
BOKORI-BROWN, MONIKA, GB;
NAYLOR, CLAIRE, GB
(73) Propriétaire/Owner:
UNIVERSITY OF EXETER, GB
(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

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(54) Title: EPSILON TOXIN EPITOPES FROM CLOSTRIDIUM PERFRINGENS WITH REDUCED TOXICITY

(57) Abrégé/Abstract:

There is provided an epsilon toxin epitope polypeptide comprising a sequence of at least 10 contiguous amino acids from SEQ ID NO:3, the sequence comprising a mutation of at least one tyrosine residue compared to the equivalent sequence in SEQ ID NO:3, the polypeptide being capable of binding an antibody which binds to SEQ ID NO:5 and having reduced toxicity compared with the toxicity of SEQ ID NO:5. The polypeptide is useful in a method of vaccinating a subject against developing a disease caused by Clostridium perfringens and/or caused by (or associated with the presence of) active epsilon toxin.



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- (71) Applicants: **UNIVERSITY OF EXETER** [GB/GB]; Northcote House, The Queen's Drive, Exeter EX4 4QJ (GB). **BIRKBECK, UNIVERSITY OF LONDON** [GB/GB]; Malet Street, Bloomsbury, London WC1E 7HX (GB).
- (72) Inventors: **TITBALL, Richard W.**; c/o University of Exeter, Northcote House, The Queen's Drive, Exeter, Devon EX4 4QJ (GB). **BOKORI-BROWN, Monika**; c/o University of Exeter, Northcote House, The Queen's Drive, Exeter, Devon EX4 4QJ (GB). **NAYLOR, Claire**; c/o Birkbeck, University of London, Malet Street, Bloomsbury, London, Greater London WC1E 7HX (GB).
- (74) Agent: **WALLIS, Naomi Rachel**; Greaves Brewster LLP, Copa House, Station Road, Cheddar, Somerset BS27 3AH (GB).

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EPSILON TOXIN EPITOPES FROM CLOSTRIDIUM PERFRINGENS WITH REDUCED TOXICITY

Field of the invention

The invention relates to novel polypeptides useful as a vaccine against diseases caused by or associated with the epsilon toxin of *Clostridium perfringens*, particularly
5 in animals susceptible to development of enterotoxemia.

Background

The rod-shaped, spore-forming, Gram-negative, anaerobe bacterium *Clostridium perfringens* is able to produce at least 17 toxins, making *C. perfringens* one of the most pathogenic species in the *Clostridium* genus. Depending on its ability to produce
10 the four typing toxins, namely α -, β -, ϵ -, and ι -toxin, *C. perfringens* strains are classified into one of five toxinotypes, referred to as types A-E (Petit *et al.* (1999) Trends Microbiol. vol. 7, 104-110).

In addition to the typing toxins, the bacterium is able to produce a variety of so called minor toxins such as β_1 , β_2 , δ , θ , λ , μ , ν , and enterotoxin (Rood (1998) Annu. Rev.
15 Microbiol. vol. 52, 333-360). Epsilon toxin (Etx) is produced by toxinotypes B and D. These strains are responsible for a severe disease called enterotoxemia, which affects predominantly sheep and lambs but also causes infections in other ruminant species, including goats and calves (Songer (1996) Clin. Microbiol. Rev. vol. 9, 216-234). Enterotoxemia in naturally infected animals is usually characterised by systemic
20 lesions in sheep and enterocolitis in goats.

The most important factor in initiating disease is overeating rich food, resulting in the presence of high amounts of carbohydrates in the intestine. This leads to disruption of the microbial balance in the gut, leading to proliferation of *C. perfringens* and consequent overproduction of Etx. The toxin causes an increase in intestinal
25 permeability, facilitating its entry into the bloodstream and allowing its dissemination to the main target organs of the kidneys and the brain (McDonel (1980) Pharmacol Ther 10(3): 617-655). Here, intoxication results in fluid accumulation due to increased permeability of blood vessels. Accumulation in the central nervous system results in neurological disorder rapidly leading to death (Finnie (2003) Aust. Vet. J. vol. 81,
30 219-221).

Etx is expressed with a signal sequence that directs export of the prototoxin from the bacterium (McDonel (1986) in *Pharmacology of bacterial toxins* eds. Dorner & Drew, Pergamon Press, 477-517). In development of disease, the relatively inactive prototoxin is converted to the active toxin by proteolytic cleavage in the gut lumen, either by digestive proteases of the host, such as trypsin and chymotrypsin (Bhown & Habeeb (1977) *Biochem. Biophys. Res. Commun.* vol. 78, 889-896), or by *C. perfringens* λ -protease (Minami *et al.* (1997) *Microbiol. Immun.* vol. 41, 527-535). Proteolytic activation of Etx can also be achieved *in vitro* by controlled proteolysis (Hunter *et al.* (1992) *Infect. Immun.* vol. 60, 102-110). Depending on the protease, proteolytic cleavage results in the removal of 10-13 amino-terminal and 22-29 carboxy-terminal amino acids (Bhown & Habeeb (1977); Minami *et al.* (1997)). Maximal activation occurs when both N- and C-termini are cleaved (Worthington & Mulders (1977) *Infect. Immun.* vol. 18, 549-551).

The 3D structure of Etx has been determined (Cole *et al.* (2004) *Nature Structural & Molecular Biology* vol. 11, 797-798) and reveals a molecule composed mainly of β -sheets, which can be divided into three functional domains. Domain I at the N-terminus contains the suggested receptor interaction region. Domain II in the middle contains an amphipathic β -hairpin, which is predicted to play a role in membrane insertion. Domain III at the C-terminus contains the C-terminal peptide, which has to be removed for activation to occur.

Epsilon toxin is an aerolysin-like β -pore forming toxin (β -PFT), with the amphipathic β -hairpin loops inserting into the membrane to form β -barrel structures. The overall fold of Etx shows similarity to the structure of aerolysin from the Gram-negative bacterium *Aeromonas hydrophila* (Parler *et al.* (1994) *Nature* vol. 367, 292-295), to parasporin-2 (PS) from *Bacillus thuringiensis* (Akiba *et al.* (2009) *J. Mol. Biol.* vol. 386, 121-133) and to a pore-forming lectin, LSL, from *Laetiporus sulphureus* (Mancheno *et al.* (2005) *J. Biol. Chem.* vol. 280, 17251-17259). The structural similarities between these toxins are most striking in their two C-terminal domains. Their N-terminal domains show a greater structural variation, which is likely to account for their differences in target cell specificities and potencies (Bokori-Brown *et al.* (2011) *FEBS J.* vol. 278, 4589-4601).

In aerolysin, the two amino-terminal domains (Domains I-II) are thought to play a role in binding to cell surfaces with overlapping functions (MacKenzie *et al.* (1999) J. Biol. Chem. vol, 274, 22604-22609) and it has been suggested that domain I of Etx, which is equivalent to domain II of aerolysin, performs a similar function (Cole *et al.* (2004)), but this has yet to be demonstrated. Domain II of aerolysin contains the mannose 6-phosphate binding loops. However, the residues of domain II involved in mannose-6-phosphate binding in aerolysin are not conserved in domain I of Etx, suggesting that the structural variation in the N-terminal receptor binding domains of these toxins is likely to account for the differences between their target cell specificities.

Etx is unique among β -PFTs because it is highly potent and has high cell specificity. Because of its high potency, Etx is considered to be a potential biological weapon for international terrorism by the U.S. Government Centres for Disease Control and Prevention (Morbidity and Mortality Weekly Report (MMWR) Recommendations and Reports (2000) vol. 49, 1-14). The 50% lethal dose (LD₅₀) of Etx in mice after intravenous injection is typically 100 ng/kg (Gill (1982) Microbiol. Rev. vol. 46, 86-94), making Etx the most potent clostridial toxin after botulinum neurotoxin. Etx also shows high cell specificity. Among the many cell lines tested, only four have been identified to be susceptible to the toxin. These include kidney cell lines of dog (MDCK (Knight *et al.* (1990) Biologicals vol. 18, 263-270)), mouse (mpkCCDcl4 (Chassin *et al.* (2007) Am. J. Physiol. Renal Physiol. vol. 293, F927-937)) and human (G-402 (Shortt *et al.* (2000) Hum. Exp. Toxicol. vol. 19, 108-116) and ACHN (Ivie *et al.* (2011) PloS ONE vol. 6, e17787)) origin. Most *in vitro* studies on Etx have been carried out using the Madin–Darby Canine Kidney (MDCK) cell line, as this cell line is the most susceptible to the toxin (Payne *et al.* (1994) FEMS Microbiol. Lett. vol. 116, 161-167). The dose of Etx to kill 50% of MDCK cells (CT₅₀) is reported to be as low as 15 ng/ml.

The binding of Etx to MDCK cells is associated with the formation of a stable, high molecular weight complex (Petit *et al.* (1997) J. Bacteriol. vol. 179, 6480-6487). Intoxicated cells undergo morphological changes that include swelling and membrane blebbing before cell death (Petit *et al.* (1997) J. Bacteriol. vol. 179, 6480-6487). The rapid toxin-induced cell death and the specificity of epsilon toxin for only a few cell

lines suggest the presence of a specific receptor(s) on target cells. The mechanism of Etx binding to cells is not known, but chemical modification studies of Etx have previously indicated that a tyrosine residue is necessary for binding of the toxin to target cells (Sakurai & Nagahama (1987) *Toxicon* vol. 25, 279-284). Toxicity appears to be a consequence of the formation of pores in the target cell membrane (Petit *et al.* (2001) *J. Biol. Chem.* vol. 276, 15736-15740).

Since progression of enterotoxemia from onset to death can be rapid in agricultural animals and given the potential for Etx to be used as a biological weapon, there is a need to identify molecules with potential for use as a vaccine against disease caused by or associated with the presence of Etx and/or caused by infection by *C. perfringens*.

Summary of Invention

According to a first aspect of the invention, there is provided an epsilon toxin epitope polypeptide comprising a sequence of at least 10 contiguous amino acids from SEQ ID NO:3, the contiguous sequence comprising a mutation of at least one tyrosine residue compared to the equivalent sequence in SEQ ID NO:3, the polypeptide being capable of binding an antibody which binds to SEQ ID NO:5 (activated wild-type *C. perfringens* epsilon toxin) and/or SEQ ID NO:22 (a reduced toxicity variant of SEQ ID NO:5) and having reduced toxicity compared with the toxicity of SEQ ID NO:5.

As stated in Table 3 below, SEQ ID NO:2 is full length wild type *C. perfringens* epsilon toxin and SEQ ID NO:3 is the same sequence, but lacking the first 32 amino acids. This sequence SEQ ID NO:3 is the sequence published for the crystal structure (see the Research Collaboratory for Structural Bioinformatics (RCSB) databank at www.rcsb.org/pdb; PDB ID: 1UYJ) and numbering of tyrosine residues referred to herein is on the basis of this sequence. SEQ ID NO:1 shows 100% sequence identity to SEQ ID NO:3 from SEQ ID NO:1 residue 33 onwards. Therefore, SEQ ID NOs:1 and 3 have 90% identity at the global alignment level, when determined as outlined below.

As mentioned above, SEQ ID NO:5 is activated wild type *C. perfringens* epsilon toxin which remains after protease cleavage, with N- and C-termini removed. SEQ ID

NOs:2 and 5 have 80% identity at the global alignment level, when determined as outlined below.

5 SEQ ID NO:22 is a sequence equivalent to SEQ ID NO:5 but with a H>A mutation at position 151 of SEQ ID NO:5 (mentioned in the Examples below as the H149A mutation, with the difference in residue numbering explained below). This is a variant of the activated toxin which may be studied in the laboratory at ACGM level 2 (Oyston *et al.* (1998) Microbiol. vol. 144 (Pt 2), 333-341) and so may be more convenient practically for determining antibody binding.

10 The polypeptide according to the invention may include a sequence of at least 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 or 100 contiguous amino acids from SEQ ID NO:3, the sequence comprising a mutation of at least one tyrosine residue compared to the equivalent sequence in SEQ ID NO:3. The inclusion of a mutation within the contiguous amino acid sequence has the result that the sequence included in the polypeptide is identical to the equivalent sequence from SEQ ID NO:3, apart from a
15 tyrosine amino acid in at least one of the positions within that sequence being substituted for a different amino acid. That is, the sequences are identical apart from at the mutated position(s). Preferably, the contiguous amino acid sequence from SEQ ID NO:3 is also found in SEQ ID NO:5.

20 In some embodiments, where the contiguous amino acid sequence from SEQ ID NO:3 comprises position 162 of SEQ ID NO:3, the amino acid at this position is mutated to one other than the histamine found in SEQ ID NO:3. For example, it may be substituted by alanine. This is the H149A mutation described elsewhere herein and its inclusion would doubly ensure that the polypeptide according to the invention could be used at ACGM level 2.

25 The level of toxicity may be determined as described herein, for example by use of a MDCK.2 cell-based LDH assay. The polypeptides of the invention provide protection, when administered to a subject such as a lamb, to the subject from developing a disease (such as enterotoxemia) caused by infection by *Clostridium perfringens* and/or caused by the presence of active epsilon toxin (or associated with the presence
30 of the toxin). Such protection may be partial, whereby the probability of an individual subject within a population of developing disease is reduced, or complete, whereby

the subject will not develop disease (i.e., the probability of developing disease caused by *C. perfringens* and/or by Etx is 0%).

The term “epsilon toxin epitope polypeptide” as used herein means a polypeptide which comprises one or more (or all) epitopes of mature activated wild-type epsilon toxin (“active toxin”), as represented by SEQ ID NO:5. The term “epitope” refers to the amino acids (typically a group of around 5 or more amino acids) within a polypeptide sequence which are essential in the generation of an immune response. These amino acids can be consecutive in the sequence but, more typically, are non-consecutive, grouping together when the tertiary structure of the native protein is formed. Provided that these amino acids are within a polypeptide environment which enables them to form the correct epitopic tertiary structure, they can be used to provide a protective vaccine composition. For example, an epsilon toxin epitope polypeptide may be one which is capable of binding to an antibody which binds to the mature activated wild-type epsilon toxin having sequence SEQ ID NO:5 and/or to the low-toxicity variant sequence SEQ ID NO:22.

In the present study, the inventors investigated the role of domain I of Etx in cell binding, to identify the key residues involved in this. This allowed them to determine the requirement for toxicity. For this, they generated site directed mutants by replacing tyrosine residues with alanine. Cytotoxicity and binding studies of these single tyrosine mutants indicated that a cluster of surface exposed tyrosines in domain I is critical for the interaction of Etx with the receptor on MDCK.2 cells. Reduced cytotoxicity of the mutants correlated with their reduced ability to bind to MDCK.2 cells. To further evaluate the roles of tyrosine residues in receptor binding, the inventors generated a double tyrosine mutant of Etx, Y43A/Y209A. Replacement of Y43 and Y209 with alanine resulted in cytotoxicity equivalent to that of inactive wild-type prototoxin, indicating that Y43A/Y209A is inactive. This apparent loss of cytotoxic activity related to the inability of Y43A/Y209A to bind to MDCK.2 cells.

Therefore, the mutated tyrosine residue in the epsilon toxin epitope polypeptide of the invention may be one or more within loop 1 (residues K32-I55, residue number according to equivalent positions in SEQ ID NO:3, as explained above) or loop 2 (S201-S220) of domain I of Etx. The mutated tyrosine residue may be, for example, one or more of Y29, Y33, Y42, Y43, Y49 and/or Y209, residue numbering being

according to the equivalent positions in SEQ ID NO:3. In a particular embodiment, the polypeptide comprises mutation of two tyrosine residues compared to SEQ ID NO:3, Y43 and Y209. The polypeptide may comprise an alanine at each of these residue positions. Alternatively or additionally to the ability to bind an antibody which
5 binds to SEQ ID NO:5 and/or SEQ ID NO:22, the polypeptide may bind an antibody which binds to SEQ ID NO:2, the non-activated prototoxin form of the epsilon toxin.

The polypeptide according to the invention may comprise the amino acid sequence RMEKYXPNAM (SEQ ID NO:33) and/or the amino acid sequence YNYLKRMEKYXPNAMAYFDK (SEQ ID NO:34), wherein "X" is any amino acid
10 other than Y. The polypeptide according to the invention may comprise the amino acid sequence GEIPSXLAFP (SEQ ID NO:35) and/or the amino acid sequence SGSEWGEIPSXLAFPRDGYK (SEQ ID NO:36), wherein "X" is any amino acid other than Y. "X" may be amino acid alanine. The polypeptide according to the invention may comprise all of SEQ ID NOs:33-36.

15 The polypeptide according to the invention may form part of a fusion protein. The polypeptide may have at least about 60% sequence identity to SEQ ID NO:6 (activated toxin sequence comprising Y43A and Y209A mutations) or SEQ ID NO:25 (activated recombinant toxin comprising Y43A, Y209A and H149A mutations) and comprising a mutation at position 43 and position 209 (as compared to the equivalent
20 positions in SEQ ID NO:3), such that a tyrosine residue at each of those positions is replaced by another amino acid such as alanine. That is, the polypeptide is at least about 60% identical to SEQ ID NO:6 (or SEQ ID NO:25) and comprises any amino acid other than tyrosine at the position equivalent to positions 32 and 198 of SEQ ID NO:6 (or positions 32 and 198 of SEQ ID NO:25), when aligned with SEQ ID NO:6
25 (or SEQ ID NO:25) using a global sequence alignment tool as described below. In another embodiment, the polypeptide according to the invention comprises an amino acid sequence at least about 60% identical to SEQ ID NO:1 (full-length wild type toxin sequence) and comprising a mutation at position 43 and position 209 (as compared to the equivalent positions in SEQ ID NO:3), such that a tyrosine residue at
30 each of those positions is replaced by another amino acid such as alanine. That is, the polypeptide is at least about 60% identical to SEQ ID NO:1 and comprises any amino acid other than tyrosine at the position equivalent to positions 75 and 241 of SEQ ID

NO:1, when aligned with SEQ ID NO:1 using a global sequence alignment tool as described below.

The polypeptide may have equal or similar toxicity to SEQ ID NO:22 and/or be non-toxic.

- 5 The polypeptide may have at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or about 99% sequence identity to SEQ ID NOs:1, 4, 6, 15, 19 or 25. The epsilon toxin epitope polypeptide may consist of or may comprise an amino acid sequence selected from SEQ ID NOs:1, 4, 6, 15, 19 or 25 (i.e., sequences comprising at least the Y43A and Y209A mutations, some also
10 comprising the H149A mutation).

As outlined further below, sequence identity may be determined using the Needleman-Wunsch Global Sequence Alignment Tool available from the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA, for example via <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, using default parameter settings.

- 15 When comparing the level of sequence identity to (for example) SEQ ID NO:6, this typically should be done relative to the whole length of SEQ ID NO:6, to avoid short regions of high identity overlap resulting in a high overall assessment of identity (i.e., a global alignment method is used). For example, a short polypeptide fragment having, for example, five amino acids might have a 100% identical sequence to a five
20 amino acid region within the whole of SEQ ID NO:6, but this does not provide a 100% amino acid identity unless the fragment forms part of a longer sequence which also has identical amino acids at the other positions equivalent to positions in SEQ ID NO:6.

- Therefore, the skilled person is readily able to determine equivalent positions between
25 two sequences, by aligning sequences to achieve maximum identical amino acids at as many positions as possible, for example by using a global sequence alignment program such as is available via <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, discussed further below.

- An epitope polypeptide according to the invention may be, as mentioned above, any
30 which comprises at least one epitope of epsilon toxin and is capable of binding an antibody which will bind to a polypeptide having sequence SEQ ID NO:5 and/or 22.

Therefore, the polypeptide may be as little as about 20 amino acids in length provided that it still binds to such an antibody, for example, it may be at least about 30, 40, 50, 70, 90, 120, 150 or about 170 amino acids in length. In some embodiments, the polypeptide may be at least about 190 amino acids in length, for example, it may be
5 between 190 and 360 amino acids in length, such as between 200-350, 220-340 or 250-330 in length. In some embodiments, the polypeptide may be at least about 200 amino acids in length, for example, at least about 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or about 330 amino acids in length. In certain specific embodiments, the polypeptide may be 263, 293, 315 or 328 amino acids in length (the
10 lengths of SEQ ID NO:6, 28, 15 and 1, respectively).

The present invention also encompasses polypeptides comprising variants of the epitope polypeptides and methods utilising these variant polypeptides. As used herein, a “variant” means a polypeptide in which the amino acid sequence differs from the base sequence from which it is derived in that one or more amino acids within the
15 sequence are substituted for other amino acids. The variant is a functional variant, in that the functional characteristics of the polypeptide from which the variant is derived are maintained. For example, the variant polypeptide may have a similar ability to bind an antibody capable of binding to a non-variant polypeptide (such as, by way of non-limiting example, SEQ ID NOs:1, 4, 6, 15, 19, 25). In particular, any amino acid
20 substitutions, additions or deletions must not alter or significantly alter the tertiary structure of one or more epitopes contained within the polypeptide from which the variant is derived, so that the variant polypeptide retains the ability to bind to an antibody which binds to SEQ ID NO:5 or 22. The skilled person is readily able to determine appropriate functional variants and to determine the tertiary structure of an
25 epitope and any alterations thereof, without the application of inventive skill.

Amino acid substitutions may be regarded as “conservative” where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type.

30 By “conservative substitution” is meant the substitution of an amino acid by another amino acid of the same class, in which the classes are defined as follows:

<u>Class</u>	<u>Amino acid examples</u>
Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic:	Asp, Glu
5 Basic:	Lys, Arg, His.

As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that polypeptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the polypeptide's conformation.

As mentioned above, non-conservative substitutions are possible provided that these do not disrupt the tertiary structure of an epitope within the polypeptide, for example, which do not interrupt the immunogenicity (for example, the antigenicity) of the polypeptide.

Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. As mentioned above, variants may suitably be at least about 60% identical to the base sequence.

As already briefly mentioned, sequence identity between amino acid sequences can be determined by comparing an alignment of the sequences. When an equivalent position in the compared sequences is occupied by the same amino acid, then the molecules are identical at that position. Scoring an alignment as a percentage of identity is a function of the number of identical amino acids at positions shared by the compared sequences. When comparing sequences, optimal alignments may require gaps to be introduced into one or more of the sequences, to take into consideration possible insertions and deletions in the sequences. Sequence comparison methods may employ gap penalties so that, for the same number of identical molecules in sequences being compared, a sequence alignment with as few gaps as possible, reflecting higher relatedness between the two compared sequences, will achieve a higher score than one with many gaps. Calculation of maximum percent identity involves the production of an optimal alignment, taking into consideration gap penalties. As mentioned above, the percentage sequence identity may be determined using the Needleman-Wunsch

Global Sequence Alignment tool, publicly available via <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, using default parameter settings. The Needleman-Wunsch algorithm was published in J. Mol. Biol. (1970) vol. 48:443-53.

A further aspect of the invention provides a polynucleotide having a nucleic acid sequence which encodes for a polypeptide according to the first aspect of the invention. Examples are provided herein as SEQ ID NOs:55-60, which encode amino acid sequences SEQ ID NOs: 1, 4, 6, 15, 19 and 25, respectively, as shown in Table 4.

The invention also encompasses variant nucleic acids encoding the polypeptides of the invention. The term "variant" in relation to a nucleic acid sequence means any

substitution of, variation of, modification of, deletion of, or addition of one or more nucleic acid(s) from or to a polynucleotide sequence, providing the resultant polypeptide sequence encoded by the polynucleotide exhibits at least the same properties as the polypeptide encoded by the basic sequence. The term therefore includes allelic variants and also includes a polynucleotide (a "probe sequence")

which substantially hybridises to the polynucleotide sequence of the present invention. Such hybridisation may occur at or between low and high stringency conditions. In general terms, low stringency conditions can be defined as hybridisation in which the washing step takes place in a 0.330-0.825 M NaCl buffer solution at a temperature of about 40-48°C below the calculated or actual melting temperature (T_m) of the probe sequence (for example, about ambient laboratory temperature to about 55°C), while high stringency conditions involve a wash in a 0.0165-0.0330 M NaCl buffer solution at a temperature of about 5-10°C below the calculated or actual T_m of the probe sequence (for example, about 65°C). The buffer solution may, for example, be SSC buffer (0.15M NaCl and 0.015M tri-sodium citrate), with the low stringency wash taking place in 3 x SSC buffer and the high stringency wash taking place in 0.1 x SSC buffer. Steps involved in hybridisation of nucleic acid sequences have been described for example in Sambrook *et al.* (2001; "Molecular Cloning: a laboratory manual", 3rd Edition, Cold Spring Harbor Laboratory Press, New York).

Polypeptides and nucleic acids of the invention may be prepared synthetically using conventional synthesisers. Alternatively, they may be produced using recombinant DNA technology and may be incorporated into suitable expression vector, which is

then used to transform a suitable host cell, such as a prokaryotic cell such as *E. coli*. The transformed host cells are cultured and the polypeptide isolated therefrom.

Therefore, the invention also provides a vector comprising such a polynucleotide. This includes recombinant constructs comprising one or more of the nucleic acid molecules described above. The constructs comprise a vector, such as a plasmid or
5 viral vector, into which a nucleic acid molecule of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter operably linked to the sequence. Large numbers of suitable vectors and promoters are
10 known to those of skill in the art and are commercially available. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are also described in Sambrook, *et al.*

A further aspect of the invention provides a cell comprising any of the polypeptide, polynucleotide or vector according to the invention. For example, a suitable cell may
15 be a *Salmonella* cell, such as a *Salmonella enterica* cell, in some embodiments from the serovar *typhimurium*. The *Salmonella* may be an attenuated strain. Strains χ 8914 and χ 9241 may optionally be employed. For example, a suitable system is described in Kulkarni *et al.* (2008, Vaccine vol. 26, 4194-4203). Preferably the host cell is not a stem cell, especially not a human stem cell.

20 A further aspect of the invention provides an antibody raised against a polypeptide according to the first aspect of the invention.

A further aspect of the invention provides a subunit vaccine comprising a polypeptide according to the first aspect of the invention. For example, this may be in the form of a fusion protein and/or in the form of a recombinant viral vaccine.

25 A further aspect provides a vaccine composition which may comprise a polypeptide, polynucleotide, vector, antibody and/or subunit vaccine according to preceding aspects of the invention. The composition may further comprise excipients and/or diluents appropriate for the means by which the composition is to be administered to a subject in need of vaccination against developing disease caused by *C. perfringens*
30 and/or Etx. Selection of appropriate components is within the routine capability of the skilled person without the application of inventive activity.

For example, the vaccine composition of the invention may conveniently be formulated using a pharmaceutically acceptable excipient or diluent, such as, for example, an aqueous solvent, non-aqueous solvent, non-toxic excipient, such as a salt, preservative, buffer and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethylolate. Aqueous solvents include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the vaccine composition are adjusted according to routine skills.

Optionally, the vaccine formulation may include a carrier. Commonly used carrier molecules are bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin, mouse serum albumin, rabbit serum albumin and the like. Synthetic carriers may be used and are readily available. Means for conjugating peptides to carrier proteins are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

In certain situations, it may also be desirable to formulate the vaccine composition to comprise an adjuvant to enhance the immune response. Such adjuvants include all acceptable immunostimulatory compounds such as, for example, a cytokine, toxin, or synthetic composition. Commonly used adjuvants include aluminium hydroxide, aluminium phosphate, calcium phosphate, Freund's adjuvants and Quil-A saponin. In addition to adjuvants, it may be desirable to co-administer biologic response modifiers (BRM) with the peptide or variant or derivative to down regulate suppressor T cell activity.

Possible vehicles for administration of the vaccine formulation include liposomes. Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. Liposomes are similar in composition to cellular membranes and, as a result, liposomes generally can be administered safely and are biodegradable. Techniques for preparation of liposomes and the formulation (e.g., encapsulation) of various molecules, including peptides and oligonucleotides, with liposomes are well known.

Depending on the method of preparation, liposomes may be unilamellar or multilamellar and can vary in size with diameters ranging from 0.02 μ m to greater than 10 μ m. Liposomes can also adsorb to virtually any type of cell and then release the encapsulated agent. Alternatively, the liposome fuses with the target cell, whereby the contents of the liposome empty into the target cell. Alternatively, an absorbed liposome may be endocytosed by cells that are phagocytic. Endocytosis is followed by intralysosomal degradation of liposomal lipids and release of the encapsulated agents. In the present context, the polypeptide according to the invention can be localized on the surface of the liposome, to facilitate antigen presentation without disruption of the liposome or endocytosis. Irrespective of the mechanism or delivery, however, the result is the intracellular disposition of the associated polypeptide.

Liposomal vectors may be anionic or cationic. Anionic liposomal vectors include pH sensitive liposomes which disrupt or fuse with the endosomal membrane following endocytosis and endosome acidification. Cationic liposomes are preferred for mediating mammalian cell transfection *in vitro*, or general delivery of nucleic acids, but are used for delivery of other therapeutics, such as peptides.

Other suitable liposomes that are used in the methods of the invention include multilamellar vesicles (MLV), oligolamellar vesicles (OLV), unilamellar vesicles (UV), small unilamellar vesicles (SUV), medium-sized unilamellar vesicles (MIN), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV), multivesicular vesicles (MVV), single or oligolamellar vesicles made by reverse-phase evaporation method (REV), multilamellar vesicles made by the reverse-phase evaporation method (MLV-REV), stable plurilamellar vesicles (SPLV), frozen and thawed MLV (FATMLV), vesicles prepared by extrusion methods (VET), vesicles prepared by French press (FPV), vesicles prepared by fusion (FUV), dehydration-rehydration vesicles (DRV), and bubblesomes (BSV). Techniques for preparing these liposomes are well known in the art.

Other forms of delivery particle, for example, microspheres and the like, also are contemplated for delivery of the peptide epitopes or polyepitopes.

Alternatively, nucleic acid-based vaccines may be produced that comprise nucleic acid, such as, for example, DNA or RNA, encoding the immunologically active

peptide epitope or polypeptide and cloned into a suitable vector (e.g., vaccinia, canarypox, adenovirus, or other eukaryotic virus vector).

Alternatively, the polypeptide may be administered in the form of a cellular vaccine via the administration of autologous or allogeneic APCs or dendritic cells that have
5 been treated *in vitro* so as to present the peptide on their surface. *Salmonella enterica* or *Escherichia coli* strains harbouring mutations which reduce their virulence and allow them to colonise a host animal without causing disease might be used to deliver vaccine antigens, especially for administration to non-human animals. The bacteria used might include strains which are already used as vaccine in livestock where the
10 attenuating lesion is not fully characterised. In addition, strains in which mutations have been deliberately introduced into the bacterium to rationally attenuate virulence could be used to deliver the epsilon toxoid. Such mutations include those in the shikimate pathway, purine biosynthesis pathway, mutations in genes encoding adenylate cyclase (*cya*) and camp receptor protein (*crp*) or mutations in the *phoP* and
15 *phoQ* genes, involved in the two-component regulatory system. Other rationally mutated strains which have been studied include those with deletions in genes involved in the biosynthesis of peptidoglycan, DNA recombination and repair or DNA methylation. Bacteria which are able to normally colonise the gut but are non-pathogenic such as *Lactobacillus sp.* or *Lactococcus sp.* strains or *Bacillus subtilis*,
20 might also be used to deliver vaccine antigens. In the case of *B. subtilis*, the vaccine antigen might be expressed on the vegetative cell or the spore. The antigen might also be delivered as a naked DNA vaccine where the gene encoding the epsilon toxoid is cloned into a mammalian expression vector and expressed from a eukaryotic promoter.

25 Such an approach, for example using *Salmonella*, offers a number of advantages. First, live *Salmonella* vaccines can be given orally (the natural route of infection), enabling a non-invasive route of vaccine administration. Second, both mucosal and systemic immune responses can be elicited, which may be important for protection against infection. In addition, live attenuated *Salmonella* vaccines are able to simulate
30 both humoral and cellular immune responses that may be important for protection against disease. Finally, since *Salmonella* is genetically tractable, recombinant

Salmonella vaccines are relatively easy to develop and are also relatively cost effective to produce.

One of the most widely studied classes of attenuated *Salmonella* used as carriers of foreign antigens are auxotrophs. For example, genetically defined mutants of the *aroA* gene, encoding 5-enolpyruvylshikimate-3-phosphate synthase, have been constructed in both *S. enterica* var. Typhimurium and var. Typhi. These mutants are attenuated and immunogenic in mice. Examples of other auxotrophic mutants include *Salmonella* with deletions in the genes involved in the purine biosynthetic pathway. Another well-studied group of attenuated *Salmonella* are mutants that have defined deletions in genes involved in the regulation of *Salmonella* virulence. For example, mutations in genes encoding adenylate cyclase (*cya*) and camp receptor protein (*crp*) affect the expression of genes involved.

In one embodiment, the vaccine composition may be included in a foodstuff (i.e., a food material suitable for consumption by a human or an animal) comprising a polypeptide and/or a polynucleotide and/or a vector and/or a cell and/or a subunit vaccine and/or vaccine composition according to preceding aspects of the invention. This may, in non-limiting examples, be in the form of pellets, crumbs or a mash which may further comprise, again for example only, grain, grass and/or protein components. The composition may also be included in drinking liquids and/or administered via a spray into the atmosphere surrounding the animal which is, consequently, inhaled by the animal.

If the vaccine composition is for administration to a human subject, it may be in a form suitable for administration orally (e.g. in a dietary supplement) and/or parenterally, for example, by injection, inhalation, or by transdermal administration via a patch, lotion or gel. The particular forms outlined above are also generally useful for administration to a human subject.

A polypeptide, polynucleotide, vector, subunit vaccine, antibody and/or vaccine composition according to the invention may be for use in a method of vaccinating a subject against developing a disease caused by *Clostridium perfringens*, the disease for example involving accumulation in the subject's bloodstream of epsilon toxin which may be released by *C. perfringens*, particularly active epsilon toxin lacking the

N- and C- termini of the full length prototoxin. The invention also provides a method of vaccinating a subject against developing a disease caused by *Clostridium perfringens* and/or caused by (or associated with the presence of) the epsilon toxin, especially active toxin, the method comprising administering to a subject a protective amount of a polypeptide, polypeptide, polynucleotide, vector, subunit vaccine, antibody and/or vaccine composition according to the invention. The subject may be a human or a non-human animal. The non-human animal may be a ruminant animal, such as a sheep, pig or goat, or a bovine animal such as a domestic cow. Young animals such as lambs, piglets, kids and calves are also included.

10 A “protective amount” is an amount sufficient to induce an immune response in the subject, such that the probability of the subject developing a disease caused by *C. perfringens*, for example caused by (or associated with the presence of) epsilon toxin, especially active toxin, is reduced or removed. For example, antibodies capable of binding to SEQ ID NO:5 and/or 22 may be detectable after the administration, where
15 such antibodies were not detectable prior to the administration, or only detectable at lower concentrations than after administration.

The invention also provides a kit comprising a polypeptide, polynucleotide, vector, subunit vaccine, antibody and/or vaccine composition according to the invention, the kit having uses, for example, in methods of vaccinating a subject against developing
20 a disease caused by infection with *Clostridium perfringens*, particularly a disease caused by epsilon toxin. The kit may further comprise instructions enabling a user to carry out a method of vaccinating a subject against developing a disease caused by *Clostridium perfringens*, particularly a disease caused by epsilon toxin.

A disease caused by *Clostridium perfringens* and/or by active epsilon toxin, as
25 mentioned herein may be, for example, enterotoxemia including pre-disease symptoms such as systemic lesions and enterocolitis. Other symptoms may include oedema of the main target organs of the kidneys and brain and damage to vascular endothelial cells. The terminal phase of enterotoxaemia is characterized by severe neurological disorders that include opisthotonus, seizures and agonal struggling.

30 Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”,

mean “including but not limited to” and do not exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well
5 as singularity, unless the context requires otherwise.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following
10 examples. Generally speaking the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any
15 other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

Brief Description of the Figures

Embodiments of the invention will now be described, by way of example only, with
20 reference to Figures 1-8 in which:

Figure 1 is a schematic representation of recombinant epsilon protoxin (P-Etx), with a N-terminal PelB leader peptide in place of the 13 amino acids N-terminal peptide sequence and with a C-terminal His-tag to aid purification (the amino acid sequences around the processing sites are also shown);

25 Figure 2A shows Coomassie staining visualisation of P-Etx expression: lane 1, post-induction bacterial protein extract from cells expressing vector only, soluble fraction; lane 2, post-induction bacterial protein extract from cells expressing vector only, insoluble fraction; lane 3, post-induction bacterial protein extract from cells expressing P-Etx, soluble fraction, P-Etx showing as an intense ~35 kDa band; lane 4,

post-induction bacterial protein extract from cells expressing P-Etx, insoluble fraction; lane 5, P-Etx after affinity purification, with an arrowhead indicating the position of epsilon prototoxin;

Figure 2B shows Western blot analysis of P-Etx expression using anti-His6 antibody
5 (lanes as in Figure 2A), with the positions of molecular mass markers are shown (M);

Figure 2C shows mass spectrometry analysis showing the actual molecular weight of the prototoxin, which corresponds to the expected molecular weight of 32.79 kDa;

Figure 3A shows Coomassie staining visualisation of expression of recombinant P-Etx mutants were expressed in *E. coli* Rosetta2(DE3) cells, with insoluble (P) and
10 soluble (S) fractions of post-induction bacterial protein extracts marked; recombinant Tyr mutants are expressed mainly in the soluble fraction (S), indicated by the presence of an intense ~35 kDa band (circled);

Figure 3B shows the result of affinity purification of Tyr mutants;

Figure 3C shows purified recombinant epsilon prototoxin trypsin treated samples
15 separated by SDS-PAGE and visualized by Coomassie staining;

Figure 4 shows that tyrosine mutants exhibit reduced cytotoxic activity towards MDCK.2 cells, with data representing the means and standard deviations of three independent experiments performed in triplicates;

Figure 5 shows the effects of Tyr mutations on the binding of epsilon prototoxin to
20 MDCK.2 cells, with mutants Y43A and Y209A showing significantly reduced binding activity relative to “wild-type” prototoxin (-21-fold and -6-fold, respectively) as determined by quantifying the fluorescent signal from each well using One-Way ANOVA analysis followed by Dunnett’s post test; data represent the means and standard deviations of three independent experiments performed in triplicates.;

25 Figure 6A shows Coomassie staining visualisation of recombinant Y43A/Y209A P-Etx expression, with insoluble (P) and soluble (S) fractions of post-induction bacterial protein extracts marked, which shows recombinant Y43A/Y209A expressed mainly in the soluble fraction (S), indicated by the presence of an intense ~35 kDa band (circled);

Figure 6B shows wild-type and Y43A/Y209A purified recombinant epsilon prototoxins treated with trypsin, separated by SDS-PAGE and visualized by Coomassie staining;

Figure 7A shows trypsin-treated wild-type and Y43A/Y209A epsilon toxins alongside
5 inactivated wild-type prototoxin were added to the medium overlying MDCK cells in 96-well plates, cytotoxicity being assessed using the LDH assay; data represent the means and standard deviations of three independent experiments performed in duplicates;

Figure 7B shows detection of MDCK cells with bound purified recombinant wild-type
10 and Y43A/Y209A epsilon prototoxins; all samples were run in quadruplicates and data were analyzed by 1way ANOVA - values significantly different from control (wild-type toxin) are shown as ***P < 0.001;

Figure 8A shows that the cytotoxicity of trypsin activated Y43A/Y209A in wild-type background was identical to that of Y43A/Y209A in H149A background;

15 Figure 8B shows that the fluorescent signal of cells treated with Y43A/Y209A in wild-type background was equivalent to that of cells treated with Y43A/Y209A in H149A background and PBS;

Examples

Materials and Methods

20 *Chemicals*

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Cell culture

MDCK.2 cells (ATCC-LGC Standards, Teddington, UK) were routinely cultured in
25 Eagle's Minimum Essential Medium (EMEM) supplemented with 10% Foetal Bovine Serum Gold (PAA, Pasching, Austria) at 37°C in a humidified atmosphere of 95% air / 5% CO₂. The culture medium was replaced every 2-3 days. Cells were routinely detached by incubation in trypsin/EDTA and split as appropriate (typically 1:6 dilutions).

Cloning of recombinant epsilon prototoxin (P-Etx)

The gene encoding epsilon prototoxin, *etxD*, from *C. perfringens* Type D strain NCTC 8346 was PCR amplified and cloned into plasmid pC10 as described by Oyston *et al.* (1998). The *etxD* gene was subsequently PCR amplified from plasmid pC10 using the oligonucleotides:

5'GGAATTCCCCATGGGTAAAGCTTCTTATGATAATGT-3' (SEQ ID NO:30)

5'-ATTGCCGCTCGAGTTTTATTCCTGGTGCC-3' (SEQ ID NO:31)

These incorporated *Nco*I and *Xho*I restriction sites, respectively (restriction enzyme cleavage sites are underlined). The PCR cycle consisted of 1 min initial denaturation of template DNA at 94°C followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, followed by 10 min final extension at 72°C. The PCR amplified fragment was digested with *Nco*I and *Xho*I restriction endonucleases and cloned into the similarly digested expression vector pET-26b(+) (Merck, Darmstadt, Germany). This placed the *etxD* gene under the regulation of the bacteriophage T7 RNA polymerase and fused the N-terminal end of the prototoxin (P-Etx) without the 13 N-terminal residues (KEISNTVSNEMSK; SEQ ID NO:32) to the PelB leader peptide and the C-terminal end of the prototoxin to a polyhistidine (6 x His) affinity tag to aid purification of the protein. In addition, recombinant P-Etx contains the H149A mutation (amino acid numbering corresponds to prototoxin without the N-terminal peptide sequence, SEQ ID NO:26), generating a low toxicity variant of Etx to enable manipulation of activated toxin at containment level 2 (Oyston *et al.* (1998)). The recombinant plasmid expressing P-Etx with the H149A mutation (SEQ ID NO:16) is termed pET26-b(+)/P-Etx_H149A.

Site directed mutagenesis

Mutations were introduced into the gene encoding P-Etx using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc. Santa Clara, US) according to the manufacturer's instructions. Synthetic oligonucleotide primer pairs (Eurofins MWG Operon, Ebersberg, Germany) were used to change each tyrosine codon to an alanine codon. Primers used for site directed mutagenesis are listed in Table 2 below. To create Tyr mutants, plasmid pET26-b(+)/P-Etx_H149A served as template, unless otherwise stated. The presence of the intended mutations was verified by DNA sequencing (Source BioScience, Cambridge, UK).

Expression and purification of recombinant epsilon prototoxin (P-Etx) and its derivatives

Recombinant P-Etx and its derivatives were expressed in *E. coli* Rosetta 2 (DE3) cells (Merck, Darmstadt, Germany) grown in ZYM-5052 autoinducing media (Studier *et al.* (2005)) supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Cells (100 mL) were grown at 37°C for 2-3 h and cultured for a further 24 h at 20°C.

For protein purification, cells were harvested by centrifugation and 2 g of cell paste was extracted by 10 mL Bugbuster protein extraction reagent (Merck, Darmstadt, Germany) containing 10 µL rlysozyme (1 KU/µL) (Merck, Darmstadt, Germany) and 10 µL benzonase nuclease (25 u/µL) (Merck, Darmstadt, Germany). The cell suspension was incubated on a rotating mixer at a slow setting for 25 min at room temperature and centrifuged at 16,000 x g for 20 min at 4°C to separate soluble (supernatant) and insoluble (pellet) fractions. The supernatant was applied to a His GraviTrap column (GE Healthcare Life Sciences, Little Chalfont, UK) following the manufacturer's guidelines. In brief, His-tagged proteins were bound to the affinity column using a buffer composed of 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4. The column was washed with a buffer composed of 20 mM sodium phosphate, 500 mM NaCl, 60 mM imidazole, pH 7.4. P-Etx was eluted in a buffer composed of 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4. All purification steps were carried out at 4°C. The P-Etx containing eluate was applied to a PD-10 Desalting Column (GE Healthcare Life Sciences, Little Chalfont, UK) for buffer exchange and further sample clean up. P-Etx was eluted from PD-10 column in 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM NaCl, pH 7.4. Protein concentrations were determined using the BCA assay (Fisher Scientific UK Ltd, Loughborough, UK) and protein was stored in aliquots at 80°C.

The purity of P-Etx and its derivatives was confirmed by SDS-PAGE. Proteins were resolved by 4-12% Bis-Tris NuPAGE gels (Invitrogen Ltd., Paisley, UK) using Surelock Xcell apparatus (Invitrogen Ltd., Paisley, UK) and NuPAGE MES SDS running buffers (Invitrogen Ltd., Paisley, UK). All samples were heated prior to loading at 95°C for 5 min in NuPAGE LDS sample buffer (Invitrogen Ltd., Paisley, UK) containing 0.1 M dithiothreitol (DTT). Gels were typically run at 200 V for 45 min. After electrophoretic separation, proteins were either visualized by SimplyBlue

staining (Invitrogen Ltd., Paisley, UK) or transferred to nitrocellulose membrane using iBlot transfer stacks (Invitrogen Ltd., Paisley, UK) and iBlot apparatus (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions. His-tag specific monoclonal antibody conjugated to horseradish peroxidase (Merck, Darmstadt, Germany) was used to detect P-Etx. Prestained molecular weight standards (Merck, Darmstadt, Germany) were used as markers. Lysate from *E. coli* Rosetta 2 (DE3)/pET26-b(+) served as a negative control.

Trypsin treatment of epsilon prototoxin (P-Etx)

For cytotoxicity assays, purified P-Etx was activated with trypsin from bovine pancreas to generate the active toxin (Etx). Trypsin was prepared in PBS and added to prototoxin at 1:100 (w:w) ratio and incubated at room temperature for 30 min. Protease Inhibitor Cocktail, EDTA-Free (Fisher Scientific UK Ltd, Loughborough, UK) was added to the supernatant to inhibit residual trypsin in the samples. Conversion of the prototoxin to toxin was assessed by SDS-PAGE.

CD spectra analysis of purified recombinant epsilon prototoxin and its variants

To confirm that the Tyr mutations do not alter the secondary structure of the proteins, purified mutants were analysed by CD spectroscopy (King's College, London).

Cytotoxicity assay

The cytotoxic activity of trypsin-activated epsilon toxins towards MDCK.2 cells was determined by measuring the release of lactate dehydrogenase (LDH) from necrotic cells using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega UK, Southampton, UK) according to the manufacturer's protocol. In brief, a 2-fold dilution series of each activated toxin (ranging from 3 μ M to 1.46 nM) was prepared in PBS and added to MDCK.2 cells seeded into 96-well plate (2×10^4 cells/well). Following incubation at 37°C for 3 hours, cell culture medium (50 μ L) was harvested from sample monolayers and transferred to a fresh 96-well enzymatic assay plate and 50 μ L of reconstituted substrate mix was added to each well. The plate was incubated for 30 min at room temperature, protected from light. Absorbance was read at 490 nm using a Model 680 Microplate Reader (Bio-Rad). The toxin dose required to kill 50% of the cell monolayer (CT₅₀) was determined by nonlinear regression analysis (GraphPad). The absorbance values for each sample were normalized by subtracting

the absorbance value obtained for the culture medium from untreated cells. All experiments were performed in triplicates with three technical replicates each.

Binding assay

On-Cell Western assays were used to measure direct binding of the purified
5 prototoxin and its variants to MDCK.2 cells. The inactive prototoxin binds to the same surface cell receptors as the full active molecule and can prevent its binding and further toxicity (Petit *et al.* (1997)). Briefly, black 96-well microtiter plates were seeded with 2×10^4 MDCK.2 cells/well in EMEM medium containing 10% foetal bovine serum. To allow cells to attach, the plates were incubated overnight at 37°C in
10 a humidified atmosphere of 95% air / 5% CO₂. The next day plates were washed with phosphate-buffered saline (PBS) containing 1 mM MgCl₂ and 1 mM CaCl₂. Each affinity-purified prototoxin was added to triplicate wells containing MDCK.2 cells at a final concentration of 5 µM. Cells were incubated with prototoxin for 30 min at 37°C in a humidified atmosphere of 95% air / 5% CO₂. For background control,
15 triplicate wells were incubated with PBS only. Unbound toxin was removed by washing cell monolayers three times in PBS. Cells were then fixed with 4% formaldehyde at room temperature for 15 min. After washing of the cell monolayers with PBS three times, wells were blocked for 1.5 hours using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA). Bound protein was detected with a 1:500
20 dilution of mouse anti-His Tag monoclonal antibody (Invitrogen Ltd., Paisley, UK) and a 1:500 dilution of IRDye 800CW goat anti-mouse IgG (H + L) antibody (LI-COR Biosciences, Lincoln, NE, USA). Plates were imaged at 800 nm to detect IRDye-labeled secondary antibody using the Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). All experiments were performed in
25 triplicates with three technical replicates each.

Immunisation of rabbits

Epsilon prototoxin variant Y43A/Y209A in wild-type background was sent to Immune Systems Ltd for immunization of New Zealand White rabbits. For each injection, 1.7 mg protein was lyophilized and used to immunize three rabbits. For the
30 initial immunisation Freund's complete adjuvant was used. The remainder immunizations used Freund's incomplete adjuvant. Injections were carried out every 14 days and harvest bleed was done on day 107, 9 days after the last injection.

Results

Expression of recombinant epsilon prototoxin (P-Etx) and its variants in E. coli

Recombinant P-Etx contains the H149A mutation (numbering of this mutation corresponds to prototoxin without the N-terminal peptide, SEQ ID NO:26), which
5 makes trypsin activated toxin 6-fold less toxic to MDCK cells and 67-fold less toxic in mice (Oyston *et al.* (1998)), allowing manipulation of activated toxin at containment level 2. H149 is located in Domain III of Etx, in close proximity to the tip of the β -hairpin in Domain II, with the side-chain of His buried by the C-terminal peptide in Domain III. Therefore, the presence of this mutation is unlikely to interfere
10 with receptor binding of Etx, which is suggested to be mediated by Domain I at the N-terminus. H149A is likely to reduce cytotoxicity of Etx either by interfering with oligomerisation or with the mechanism of extension of the β -hairpin. In addition, MDCK cell assay of the H149A site directed mutant (EtxB) showed that H149 does not play a key role in Etx activity (Payne, *et al.* (1997) Rev. Med. Microbiol. vol. 8,
15 S31).

To identify residues involved in receptor binding of Etx, the gene encoding epsilon prototoxin (P-Etx) was cloned into the expression vector pET-26b(+) as described in Materials and Methods. Recombinant P-Etx contains the PelB signal peptide in place of the 13 amino acids N-terminal peptide, and a C-terminal polyhistidine (6 x His) tag
20 downstream of the C-terminal peptide sequence to aid subsequent purification of the prototoxin by affinity chromatography (Figure 1). The PelB signal peptide directs the expressed P-Etx protein to the bacterial periplasm where this sequence is removed by a signal peptidase.

For high level expression of P-Etx, recombinant plasmid was transferred into DE3
25 lysogen *E. coli* strain Rosetta 2 carrying a chromosomal copy of the bacteriophage T7 RNA polymerase gene under the control of the lacUV5 promoter, and expression was induced using the autoinduction system as described by Studier (2005; Prot. Exp. Purif. vol. 41, 207-234). As shown in Figure 2A, recombinant P-Etx protein is expressed mainly in the soluble fraction. Full length P-Etx without the PelB leader
30 peptide sequence was predicted to encode a 32.79 kDa protein. The recombinant protein had an apparent molecular size of 35 kDa (His-tagged protein) as detected by SDS-PAGE and Western blot analysis (Figures 2 A, B). His-tag affinity purification

resulted in high yields of about 100 mg purified protein per L culture. The actual molecular weight of the prototoxin was determined by mass spectrometry analysis, which corresponded to the expected molecular weight of 32.79 kDa (Figure 2 C).

Site directed mutagenesis of P-Etx to identify the role of tyrosines in receptor binding

5 To test the role of the N-terminal domain I of Etx in cell binding, the inventors initially targeted two loops in this region by deletion mutagenesis, termed loop 1 (Δ K32-I55) and loop 2 (Δ S201-S220). However, loop deletions lead to toxin instability as most of the protein expressed in the insoluble fraction and purification of soluble protein resulted in yields that were impractical for further cytotoxicity and
10 binding assays. To identify the role of loops 1 and 2 in cell binding, the inventors next concentrated on mutagenesis of individual residues within these loops. Six surface exposed tyrosines were selected for site directed mutagenesis, five residues in loop1 (Y29, Y33, Y42, Y43 and Y49) and one residue in loop2 (Y209), each being replaced individually with alanine. Each Tyr mutant was expressed and purified using the
15 method developed for wild-type P-Etx. Similar to wild-type P-Etx, all Tyr mutants expressed mostly in the soluble fraction and gave similar yields and purity to wild-type P-Etx (Figures 3A, 3B), indicating that these mutations do not affect the structure of the toxin. In addition, the trypsin digest profiles of all Tyr mutants were identical to that of wild-type P-Etx, producing a lower molecular weight active toxin (Figure 3C).
20 Further CD spectra analysis confirmed that all Tyr mutants are adopting a predominantly β -sheet conformation, similar to wild-type P-Etx (data not shown), indicating that the single tyrosine mutants are folded correctly.

Cytotoxic activity of single tyrosine mutants

Recombinant P-Etx can be activated by trypsin digestion, which removes a C-terminal
25 peptide together with the His-tag (Figure 1). To determine the effect of the single tyrosine mutations on cytotoxic activity of Etx, each mutant prototoxin was activated by trypsin digestion. A 2-fold dilution series (ranging from 3 μ M to 1.46 nM) of each activated toxin was added to MDCK.2 cells seeded into 96-well plate as described in Materials and Methods. Following 3 h incubation at 37°C, the cell culture medium
30 was harvested and cytotoxicity was measured by the LDH assay, which quantitatively measures the amount of LDH released from the cytosol of lysed cells into the cell culture medium. The tissue culture dose of each toxin that killed 50% of the cells

(CT₅₀) was determined by nonlinear regression analysis and compared to that of “wild-type” Etx (comprising the H149A mutation). Cytotoxic activity of tyrosine mutants was expressed as the fold-change in CT₅₀ relative to “wild-type” toxin. Mutants Y43A and Y209A showed significantly reduced cytotoxic activity relative to “wild-type” toxin (27-fold and 10-fold increase in CT₅₀, respectively, Figure 4). The Y43A mutation is located in loop 1. The cytotoxicity of MDCK.2 cells treated with inactivated P-Etx was indistinguishable from that of untreated control cells (data not shown). The average CT₅₀ for “wild-type” epsilon toxin was determined to be 22 nM in 100 µl (721 ng per ml).

10 *Binding of single Tyrosine mutants to MDCK.2 cells*

The inventors next tested if the reduced cytotoxicity of the single Tyr mutants related to their reduced ability to bind to MDCK.2 cells. For this, on-cell Western studies were carried out using prototoxin as described in Materials and Methods. In brief, 96-well plates seeded with 2×10^4 MDCK.2 cells/well were incubated with purified prototoxin at a final concentration of 5 µM for 30 min at 37°C. Bound protein was detected with a His-tag specific primary antibody and an IRDye 800CW-labeled secondary antibody. Plates were imaged using an Odyssey imager. All binding studies used prototoxin as it can bind to cells without causing cell lysis (see Figure 7A). Mutants Y43A and Y209A showed significantly reduced binding activity relative to ‘wild-type’ prototoxin (-21-fold and -6-fold, respectively; Figure 5) as determined by quantifying the fluorescent signal from each well using one-way ANOVA analysis. The most significant reduction in fluorescent signal was observed with the Y43A mutant, indicating that Y43 plays a critical role in cell binding. The reduced binding ability of the Tyr mutants correlated with their reduced ability to bind to cells (Table 1).

Table 1

Tyrosine mutant	% cytotoxic activity relative to wild-type	% binding activity relative to wild-type
Y33A	45	75
Y29A	32	62
Y42A	19	33
Y49A	15	26
Y209A	10	19
Y43A	4	5

The % cytotoxic activity of the tyrosine mutants is based on the CT_{50} fold-change relative to wild-type as determined in Figure 4. The % binding activity of the tyrosine mutants is based on the fluorescent signal intensity relative to wild-type as determined in Figure 5.

The double Tyr mutant Y43A/Y209A is inactive

To further evaluate the roles of the tyrosine residues in cell binding, a double Tyr mutant was generated combining the Y43A and Y209A mutations, termed Y43A/Y209A, which had the largest effect on reducing cytotoxic and binding activities of epsilon toxin. Y43A/Y209A was expressed and purified using the method developed for wild-type P-Etx. Similar to wild-type P-Etx, Y43A/Y209A expressed mostly in the soluble fraction and gave similar yields and purity to “wild-type” P-Etx (Figure 6A). In addition, its trypsin digest profile was identical to that of “wild-type” P-Etx, indicating that combining mutations Y43A and Y209A does not affect the structure of the toxin (Figure 6B). CD spectra analysis further confirmed that the double tyrosine mutant is adopting a predominantly β -sheet conformation, similar to “wild-type” P-Etx (data not shown).

To determine the effect of Y43A/Y209A on cytotoxic activity of epsilon toxin, a 2-fold dilution series (ranging from 3 μ M to 1.46 nM) of trypsin activated toxin was added to MDCK.2 cells seeded into 96-well plate as described in Materials and Methods. Following 3 h incubation at 37°C, the cell culture medium was harvested and cytotoxicity was measured using the LDH assay. The cytotoxic activity of trypsin activated Y43A/Y209A was compared to that of inactivated “wild-type” P-Etx. As shown in Figure 7A, the cytotoxicity of trypsin activated Y43A/Y209A was equivalent to that of inactivated “wild-type” P-Etx, indicating that this mutant is inactive and combining mutations Y43A and Y209A has a cumulative effect on reducing cytotoxicity of Etx. To test if the loss of cytotoxic activity of Y43A/Y209A correlated with its inability to bind to MDCK.2 cells, on-cell Western studies were carried out using inactive prototoxin as described in Materials and Methods. As shown in Figure 7B, the fluorescent signal of cells treated with Y43A/Y209A is equivalent to that of cells treated with PBS only, indicating that the apparent loss of cytotoxic activity of Y43A/Y209A is due to its inability to bind to cells.

Y43A/Y209A as vaccine candidate

The inactive Y43A/Y209A mutant identified in this study also carries the H149A mutation so the toxin can be manipulated at ACGM level 2. To exclude the possibility that the double Tyr mutant is inactive because of a cumulative effect based on the presence of the H149A mutation, H149 was re-introduced into the double Tyr mutant by site directed mutagenesis to create the double mutant in the H149 wild type background. The cytotoxic activity and binding activity of Y43A/Y209A mutant in wild-type background (Y43A/Y209A_A149H) was identical to that of in H149A background (Figure 8). This mutant was selected for further testing as a vaccine candidate.

Antiserum raised in rabbits was screened for its ability to protect cultured MDCK.2 cells against “wild-type” toxin induced cytotoxicity. Antiserum was also tested for its ability to block binding of wild-type P-Etx to MDCK.2 cells.

Discussion

The present data shows that Domain I of epsilon toxin plays a role in cell binding. The inventors' initial approach was to determine whether the loop regions in domain I are involved in cell binding, but they were unable to generate sufficient amount of soluble and stable protein from these loop deletion mutants for further assays. In contrast, all of the Tyr mutants were soluble and CD analysis indicated that these proteins were correctly folded, ruling out the possibility that the reduced cytotoxic activity of these mutants was due to their change in structure. The results confirm that domain I of Etx plays a role in cell binding and also that cytotoxicity cannot occur in the absence of binding.

Therefore, the results confirm that domain I of Etx plays a role in recognition of the cell surface. However, the identity of the cell surface receptor(s) for Etx is yet to be determined. Like aerolysin and PS, Etx appears to target the DRM domains on cell surfaces (Abrami & van Der Groot (1999) J. Cell Biol. vol. 147, 175-184). Both monomeric and heptameric Etx accumulate on these DRMs. Although the prototoxin is unable to heptamerise, it is able to bind to the same receptor as that used by activated toxin, indicating that heptamerisation is not a prerequisite for binding to target cells. It has been shown that changes in ganglioside content in DRMs affect the

binding of the toxin (Shimamoto *et al.* (2005) Microbiol. Immunol. vol. 49, 245-253). However, there is no direct evidence of toxin-binding to ganglioside.

The inventors also identified a double Tyr mutant Y43A/Y209A with apparent lack of cytotoxic and binding activity and have shown that the presence of the H149A mutation does not contribute to its loss of activity. Y43A/Y209A in wild-type background (Y43A/Y209A_A149H) could form the basis of a recombinant vaccine against Etx-mediated enterotoxemia in animals. The presence of two mutations in Y43A/Y209A greatly reduces the risk of reversion to full activity compared to a recombinant protein with only a single mutation, making Y43A/Y209A an ideal vaccine candidate. In addition, recombinant vaccines offer low batch to batch variability compared the existing commercially available vaccine against Etx that is based on treating *C. perfringens* type D culture filtrate with formaldehyde.

In conclusion, the inventors have identified a distinct cluster of surface exposed Tyrosine residues in domain I of epsilon toxin that is critical for the interaction of Etx with the receptor on MDCK.2 cells, providing further evidence that Etx binding to cells is mediated via domain I. However, the identity of the receptor on the target cell remains to be elucidated. A recombinant Y43A/Y209A variant protein was also generated, that could be used to develop a more effective vaccine against Etx-mediated enterotoxemia in animals.

Table 2 - primers used for site directed mutagenesis

Amino acid change	SEQ ID NO	Primer sequence ^a
Y29A_F	37	CTTCTTATGATAATGTAGATACATTAATTGAGAAAGGAAGAG <u>GCG</u> AAT ACAAAATATAATTACTTAAAGAGAATGGAAAAATATTA
Y29A_R	38	TAATATTTTTCCATTCTCTTTAAGTAATTATATTTTGTATT <u>CGC</u> TCTCC TTTCTCAATTAATGTATCTACATTATCATAAGAAG
Y33A_F	39	GTAGATACATTAATTGAGAAAGGAAGATATAATACAAAAG <u>GCG</u> AATTA CTTAAAGAGAATGGAAAAATATTATCCTAATGCT
Y33A_R	40	AGCATTAGGATAATATTTTTCCATTCTCTTTAAGTAATT <u>CGC</u> TTTTGTATT ATATCTTCCTTTCTCAATTAATGTATCTAC
Y42A_F	41	AAAGGAAGATATAATACAAAATATAATTACTTAAAGAGAATGGAAAA <u>AGCG</u> TATCCTAATGCTATGGCATATTTTGATAA
Y42A_R	42	TTATCAAAATATGCCATAGCATTAGGATAC <u>GCG</u> TTTTTCCATTCTCTTAA GTAATTATATTTTGTATTATATCTTCCTTT
Y43A_F2	45	GAAAGGAAGATATAATACAAAATATAATTACTTAAAGAGAATGGAAAA ATAT <u>GCG</u> CCTAATGCTATGGCATATTTTGATAAGG
Y43A_R2	46	CCTTATCAAAATATGCCATAGCATTAGG <u>GCG</u> CATATTTTTCCATTCTCTTT AAGTAATTATATTTTGTATTATATCTTCCTTTC
Y49A_F	47	GAATGGAAAAATATTATCCTAATGCTATGGCAG <u>GCG</u> TTTGATAAGGTTAC TATAAATCCACAAGGAAA
Y49A_R	48	TTTCCTTGTGGATTTATAGTAACCTTATCAAAC <u>GCG</u> TGCCATAGCATTAGG ATAATATTTTTCCATTC
Y209A_F	49	GTGAATGGGGAGAGATACCTAGT <u>GCG</u> TAGCTTTTCCTAGGGATGGTTA
Y209A_R	50	TAACCATCCCTAGGAAAAGCTAAC <u>GCG</u> ACTAGGTATCTCTCCCATTCAC
A149H_F	51	CAAATACAAATACAAATACTAATTCAAAGAAATTACTCATAATGTCCC TTCACAAGATATACTAG
A149H_R	52	CTAGTATATCTTGTGAAGGGACATTATGAGTAATTTCTTTTGAATTAGT ATTTGTATTTGTATTTG

^aUnderlined bases are the codons used for substitution. Amino acid numbering for Y mutations correspond to prototoxin with the N-terminal peptide sequence (corresponds to PDB 1UYJ; SEQ ID NO:3). Amino acid numbering for the A149H mutation corresponds to prototoxin without the N-terminal peptide sequence.

Table 3 - identity of sequences included in application

SEQ ID NO	Identity of sequence
1	full length epsilon toxin with Y43A and Y209A mutations
2	full length wild-type native epsilon toxin
3	sequence used to obtain crystal structure (PDB ID:1YUJ)
4	full length epsilon toxin with Y43A, Y209A and H149A mutations
5	trypsin activated wild-type recombinant epsilon toxin
6	trypsin activated recombinant epsilon toxin with Y43A and Y209A mutations
7	full length epsilon toxin with Y43A mutation
8	full length epsilon toxin with Y209A mutation
9	full length epsilon toxin with H149A mutation
10	full length epsilon toxin with Y43A and H149A mutations
11	full length epsilon toxin with Y209A and H149A mutations
12	full length recombinant epsilon toxin
13	full length recombinant epsilon toxin with Y43A mutation
14	full length recombinant epsilon toxin with Y209A mutation
15	full length recombinant epsilon toxin Y43A and Y209A mutation
16	full length recombinant epsilon toxin with H149A mutation
17	full length recombinant epsilon toxin with H149A and Y43A mutations
18	full length recombinant epsilon toxin with H149A and Y209A mutations
19	full length recombinant epsilon toxin with H149A, Y43A and Y209A mutations
20	trypsin activated wild-type recombinant epsilon toxin with Y43A mutation
21	trypsin activated wild-type recombinant epsilon toxin with Y209A mutation
22	trypsin activated recombinant epsilon toxin with H149A mutation
23	trypsin activated recombinant epsilon toxin with H149A and Y43A mutations
24	trypsin activated recombinant epsilon toxin with H149A and Y209A mutations
25	trypsin activated recombinant epsilon toxin with H149A, Y43A and Y209A mutations
26	native epsilon toxin without N-terminal peptide
27	native epsilon toxin without N-terminal peptide and with H149A mutation
28	recombinant epsilon toxin without PelB leader peptide
29	recombinant epsilon toxin without PelB leader peptide with H149A mutation

Table 4 – DNA sequences encoding for amino acid sequences

DNA SEQ ID NO	Encoding for amino acid SEQ ID NO
55	1
56	4
57	6
58	15
59	19
60	25

CLAIMS

1. An epsilon toxin epitope polypeptide comprising a sequence at least 260 amino acids in length comprising contiguous amino acids from SEQ ID NO:3 and comprising the amino acid sequence RMEKYXPNAM (SEQ ID NO:33), wherein "X" is any amino acid other than Y, wherein the polypeptide binds an antibody which binds to SEQ ID NO:5 and has reduced toxicity compared with the toxicity of SEQ ID NO:5.
2. The polypeptide according to claim 1 comprising a mutation at position Y29, Y33, Y42, Y49 and/or Y209, as found in SEQ ID NO:3.
3. The polypeptide according to claims 1 or 2 comprising a mutation at position Y43 and Y209, as found in SEQ ID NO:3.
4. The polypeptide according to claim 2 or 3 wherein amino acid Y is replaced with amino acid A.
5. The polypeptide according to any one of claims 1-4 comprising the amino acid sequence YNYLKRMEKYXPNAMAYFDK (SEQ ID NO:34), wherein "X" is any amino acid other than Y.
6. The polypeptide according to any one of claims 1-5 comprising the amino acid sequence GEIPSXLAFP (SEQ ID NO:35), wherein "X" is any amino acid other than Y.
7. The polypeptide according to any one of claims 1-6 comprising the amino acid sequence SGSEWGEIPSXLAFPRDGYK (SEQ ID NO:36), wherein "X" is any amino acid other than Y.
8. The polypeptide according to any one of claims 1-7 comprising all of SEQ ID NOs:33-36.
9. The polypeptide according to any one of claims 5-8 wherein "X" is amino acid alanine.
10. The polypeptide according to any one of claims 1-9 which is non-toxic.
11. The polypeptide according to any one of claims 1-10 which has at least 60% sequence identity to SEQ ID NO:6 or SEQ ID NO:25 and comprising a mutation at position 43 and position 209 as compared to the equivalent positions in SEQ ID NO:3, such that a tyrosine residue at each of those positions is replaced by another amino acid.
12. The polypeptide according to claim 11 wherein the another amino acid is alanine.
13. The polypeptide according to any one of claims 1-12 consisting of or comprising an amino acid sequence selected from SEQ ID NOs:1, 4, 6, 15, 19 or 25.
14. A polynucleotide having a nucleic acid sequence which encodes for a polypeptide according to any one of claims 1-13.

15. A polynucleotide having a nucleic acid sequence selected from SEQ ID NOs:55-60.
16. A vector comprising a polynucleotide according to claim 14 or 15.
17. A cell comprising a polypeptide according to any one of claims 1-13 and/or a polynucleotide according to claim 14 or 15 and/or a vector according to claim 16.
18. A subunit vaccine comprising a polypeptide according to any one of claims 1-13.
19. An antibody raised against a polypeptide according to any one of claims 1-13 and which specifically binds said polypeptide.
20. A composition comprising the antibody of claim 19 and a carrier.
21. The composition according to claim 20 which is a foodstuff for a human or animal.
22. An antibody according to claim 19 or a composition according to claim 20 or 21, for use in a subject against developing disease caused by *Clostridium perfringens* and/or caused by active epsilon toxin.
23. Use of a protective amount of an antibody according to claim 19 or a composition according to claim 20 or 21, in a subject against developing disease caused by *Clostridium perfringens* and/or caused by active epsilon toxin.
24. The antibody or composition according to claim 22, wherein the subject is a ruminant animal, or is a human.
25. A vaccine composition against a disease caused by *Clostridium perfringens* and/or caused by active epsilon toxin comprising a polypeptide according to any one of claims 1-13 and/or a polynucleotide according to claim 14 or 15 and/or a vector according to claim 16 and/or a cell according to claim 17 and/or a subunit vaccine according to claim 18 and further comprising a carrier.
26. A vaccine composition according to claim 25 which is a foodstuff for a human or animal.
27. A polypeptide according to any one of claims 1-13 and/or a polynucleotide according to claim 14 or 15 and/or a vector according to claim 16 and/or a cell according to claim 17 and/or a subunit vaccine according to claim 18 and/or a vaccine composition according to claim 25 or 26 for use in vaccinating a subject against developing a disease caused by *Clostridium perfringens* and/or caused by active epsilon toxin.
28. Use of a protective amount of a polypeptide according to any one of claims 1-13 and/or a polynucleotide according to claim 14 or 15 and/or a vector according to claim 16 and/or a cell according to claim 17 and/or a subunit vaccine according to claim 18 and/or a vaccine composition according to claim 25 or 26 for vaccinating a subject against developing a disease caused by *Clostridium perfringens* and/or caused by active epsilon toxin.

29. A polypeptide, polynucleotide, vector, cell and/or vaccine composition for use according to claim 27, or the use according to claim 28, wherein the subject is a ruminant animal, or is a human.
30. A kit comprising a carrier and a polypeptide according to any one of claims 1-13 and/or a polynucleotide according to claim 14 or 15 and/or a vector according to claim 16 and/or a cell according to claim 17 and/or a subunit vaccine according to claim 18 and/or an antibody according to claim 19 and/or a vaccine composition according to claim 25 or 26.

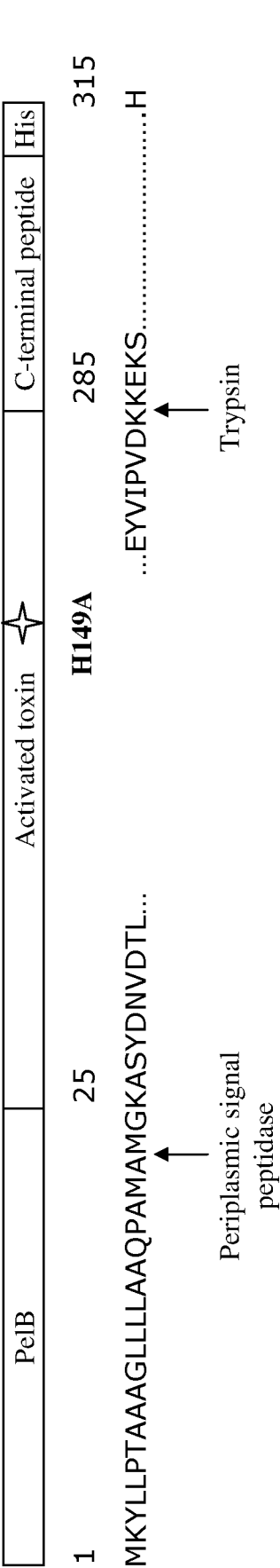
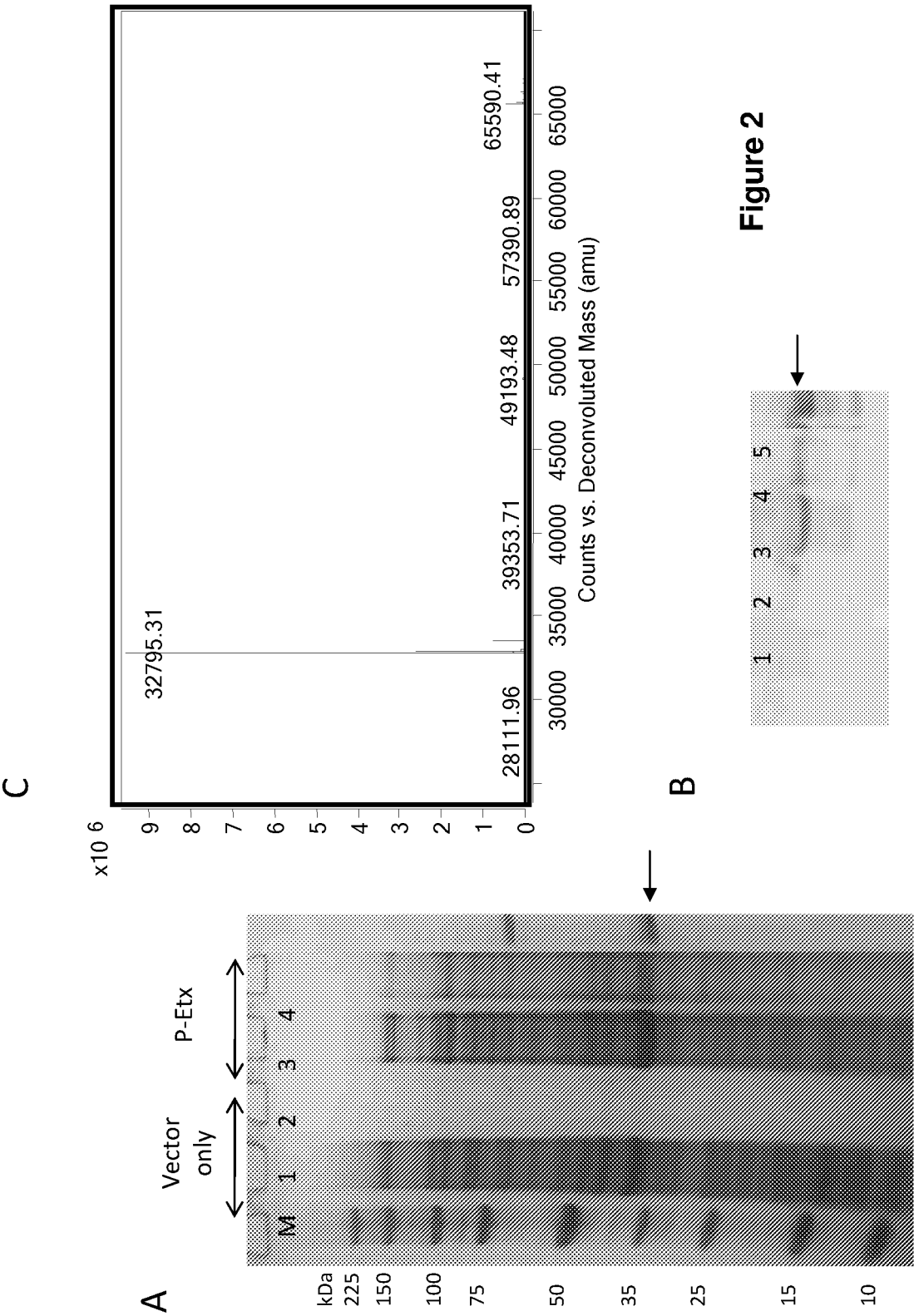
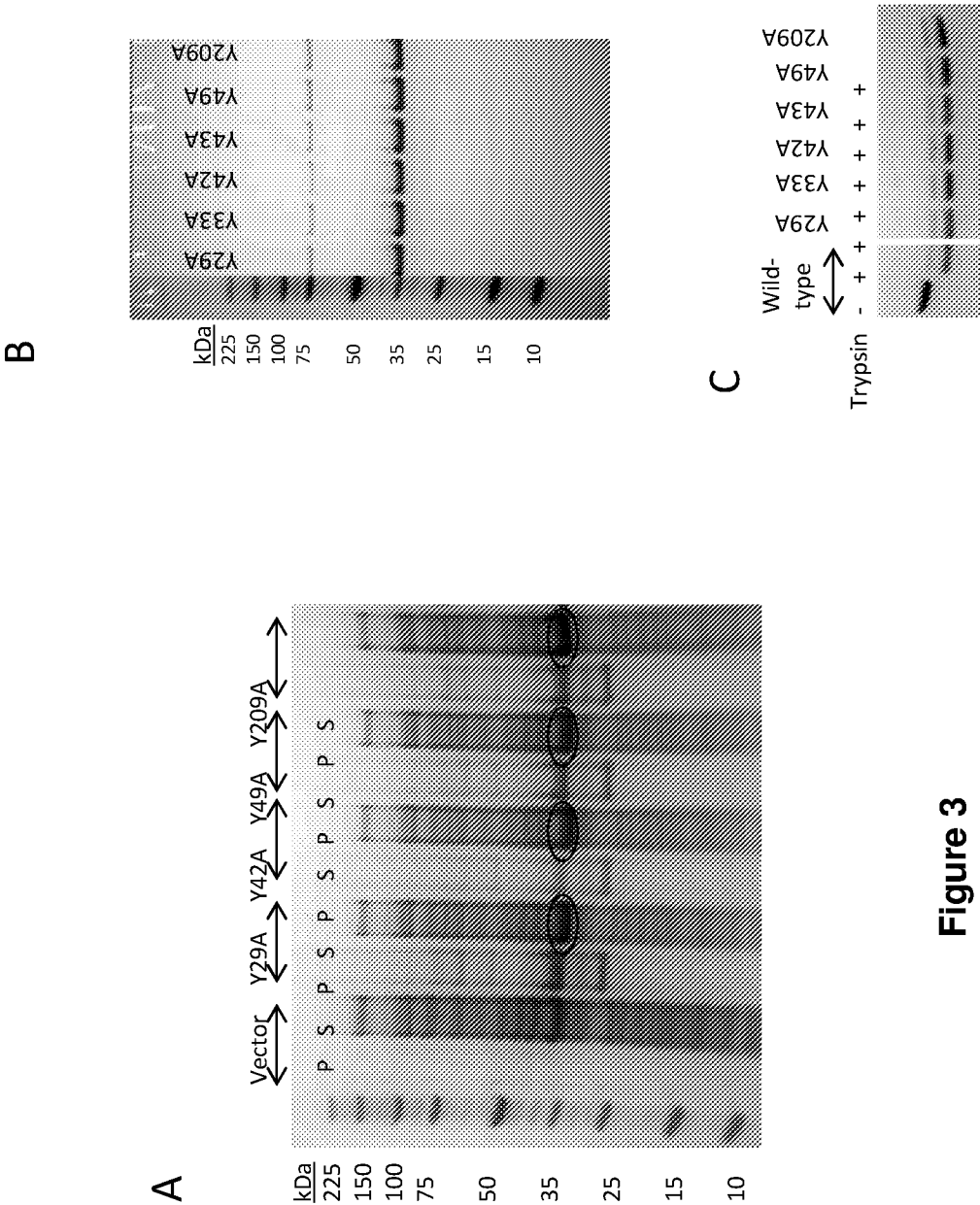


Figure 1





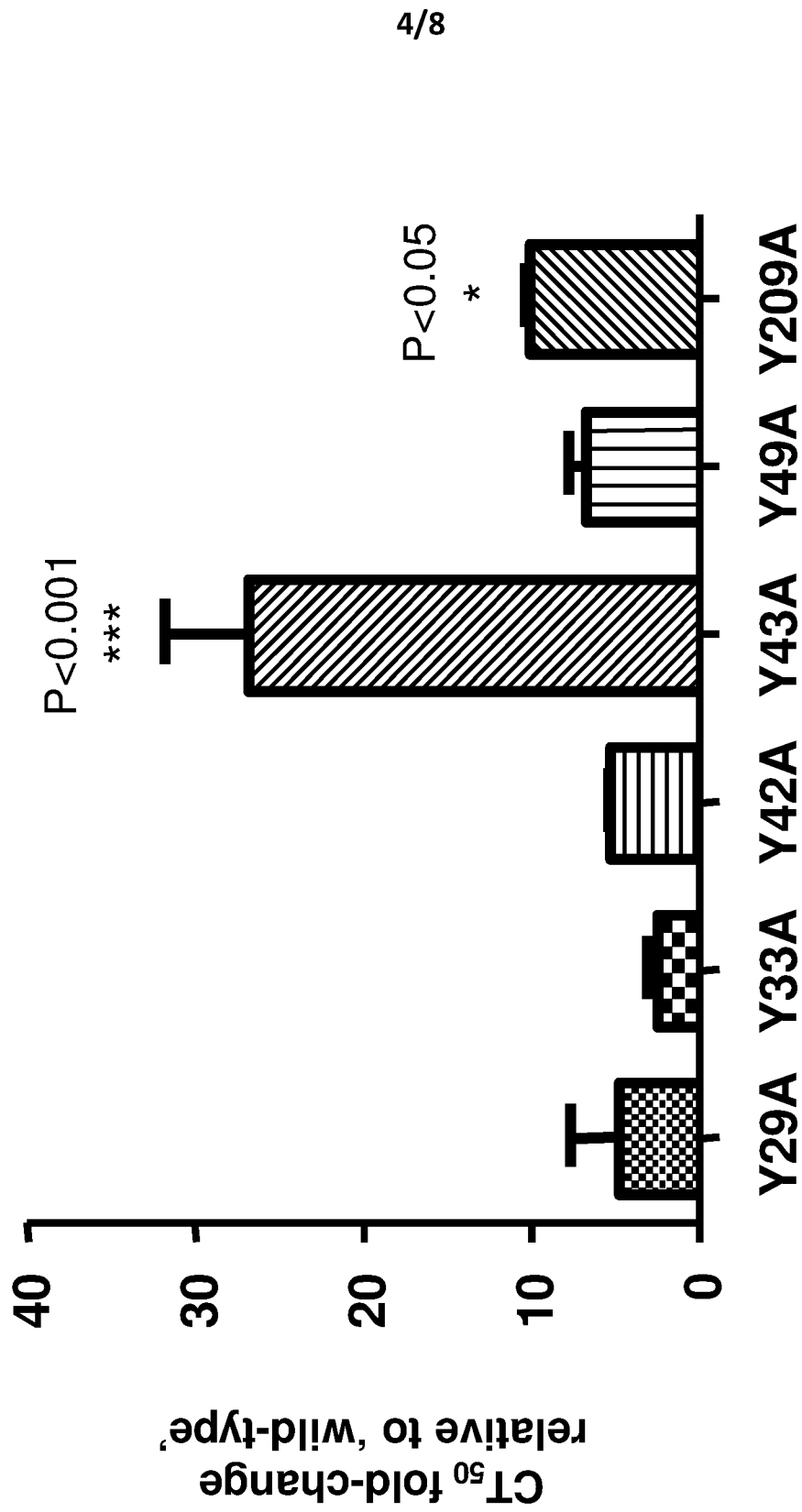


Figure 4

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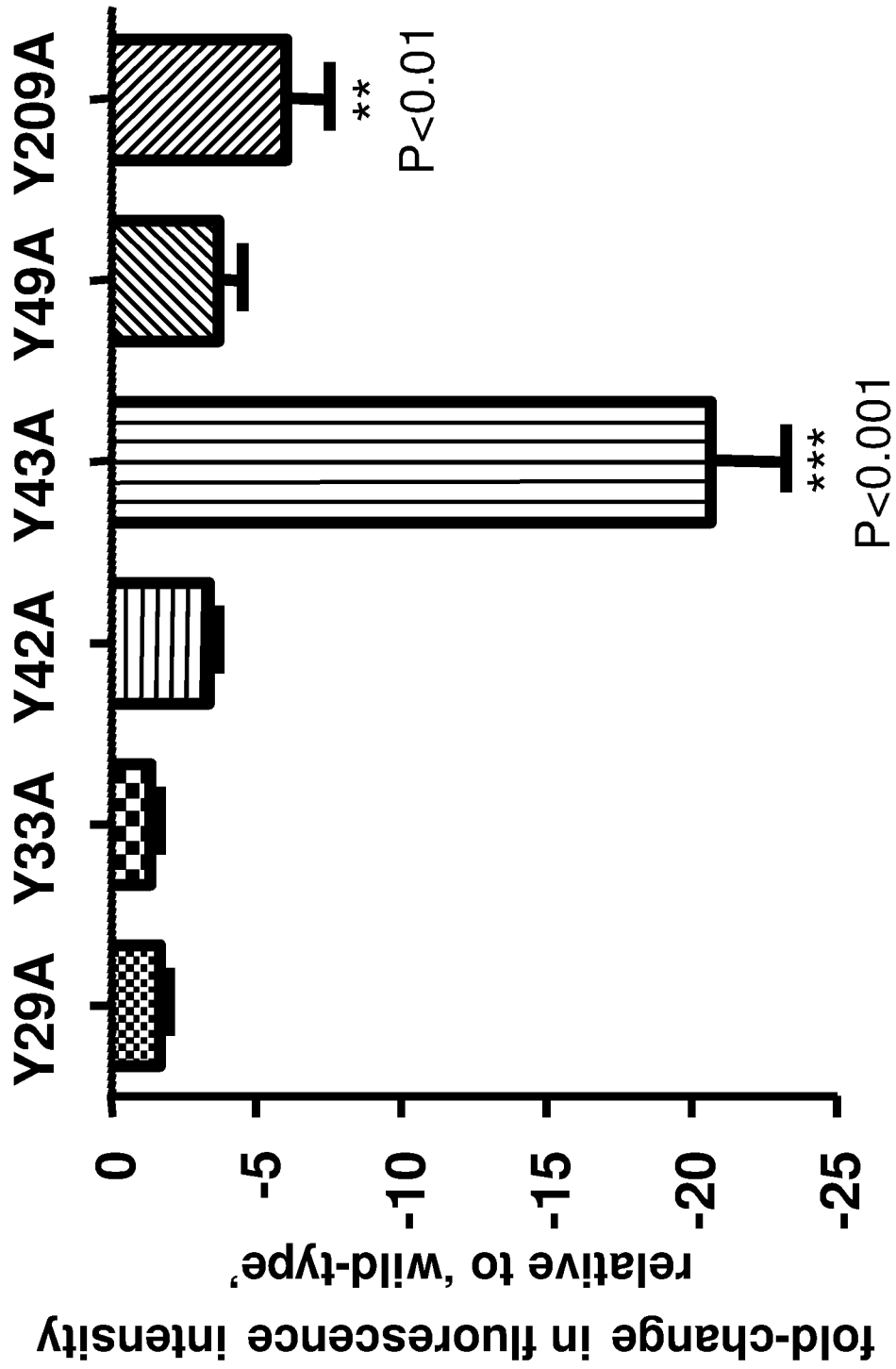
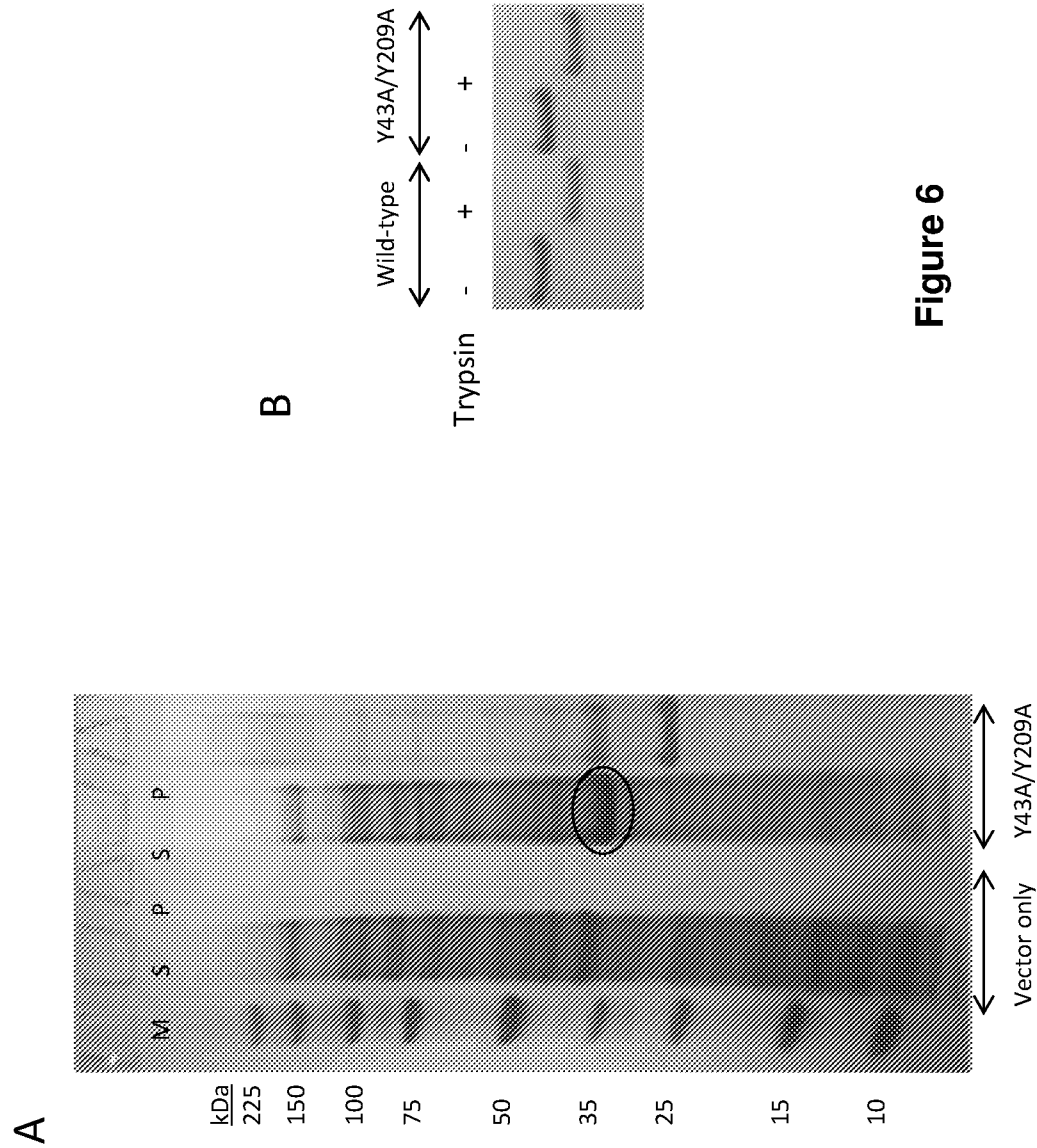


Figure 5



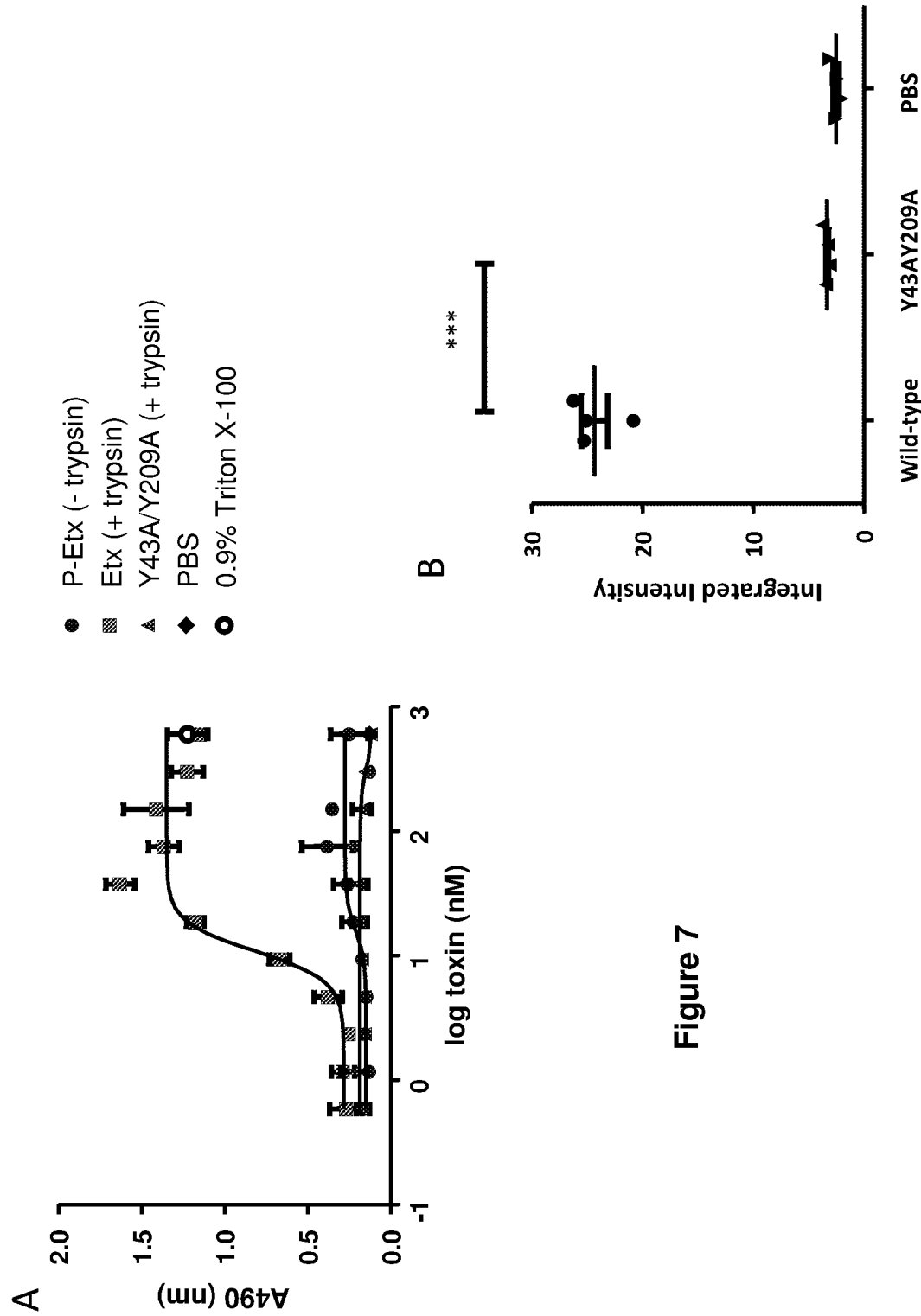


Figure 7

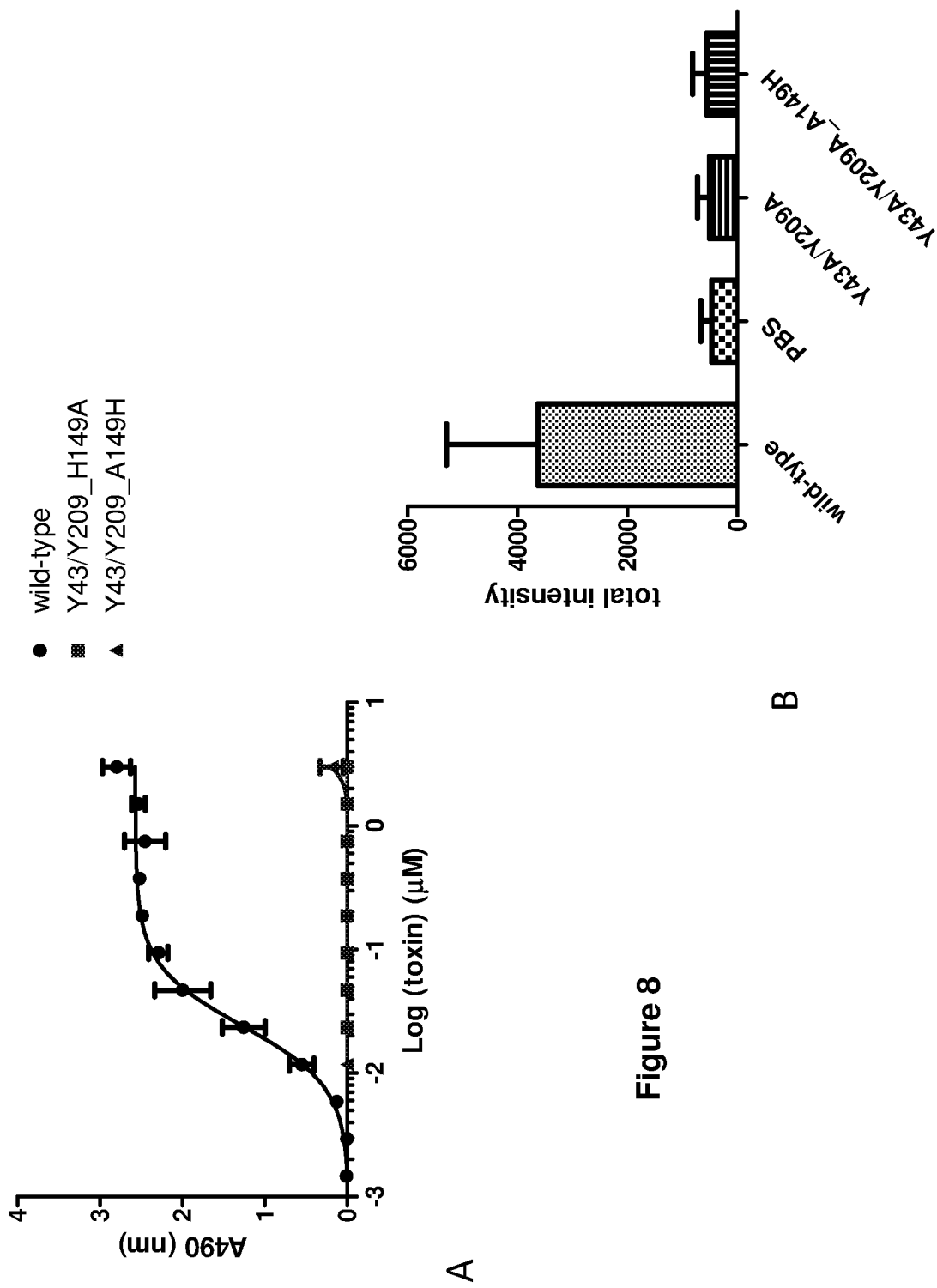


Figure 8