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(54) **MODIFIED CLOSTRIDIAL NEUROTOXINS AS VACCINES AND CONJUGATE VACCINE PLATFORMS**

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(2017.08); **C12N 15/102** (2013.01); **C07K**

2319/55 (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Provided herein are engineered non-catalytic, non-toxic tetanus toxin variants and methods of using such engineered tetanus toxin variants as low dose, protective vaccines that are non-toxic and more potent than their respective chemically inactivated toxoids. In addition, provided herein are conjugate vaccine carriers comprising engineered tetanus toxin variants and methods of using such conjugate vaccines to elicit T-cell dependent immune memory responses which can target a broad spectrum of microbial pathogens as a single vaccine.

9 Claims, 15 Drawing Sheets

Specification includes a Sequence Listing.

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FIG. 1

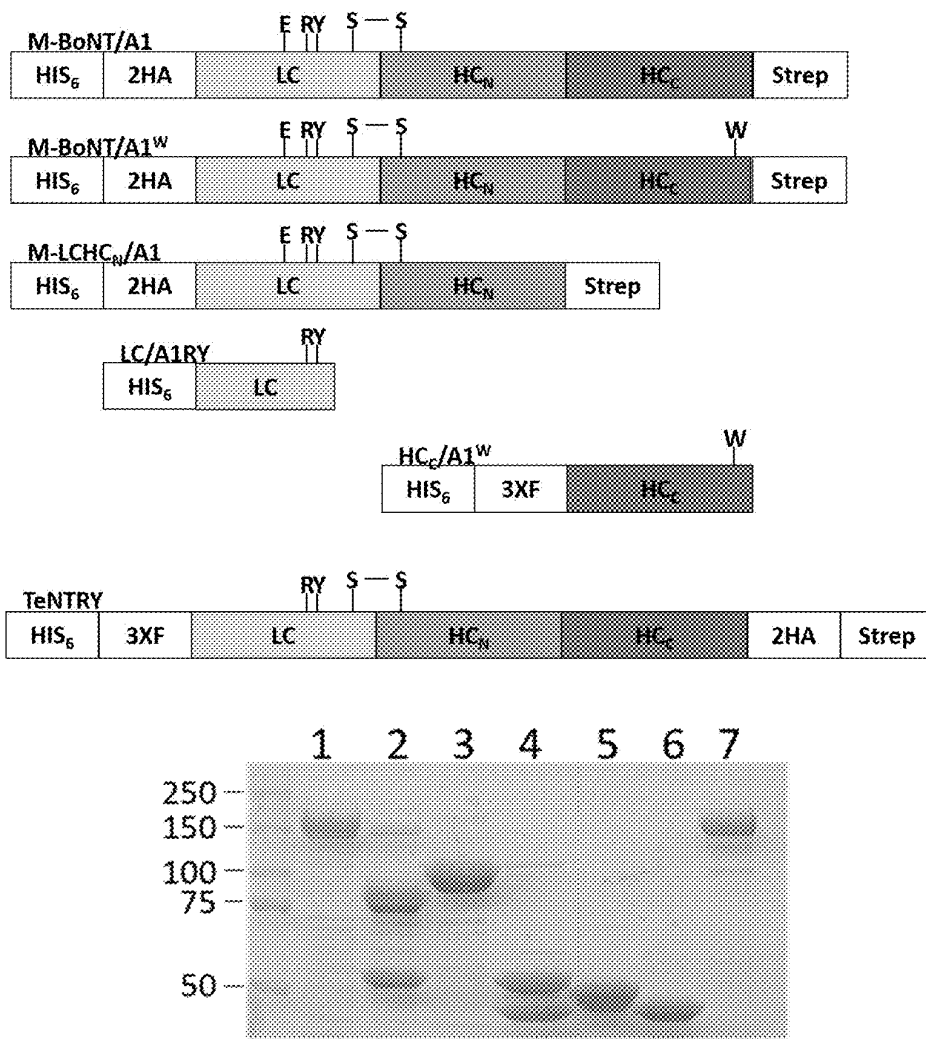
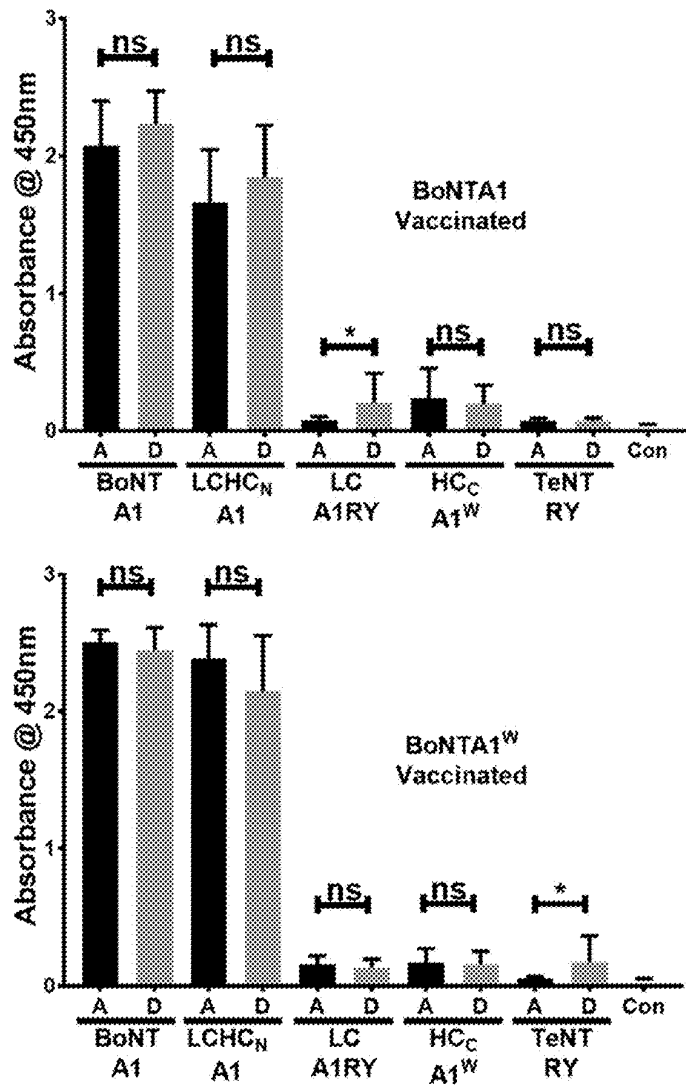


FIG. 2



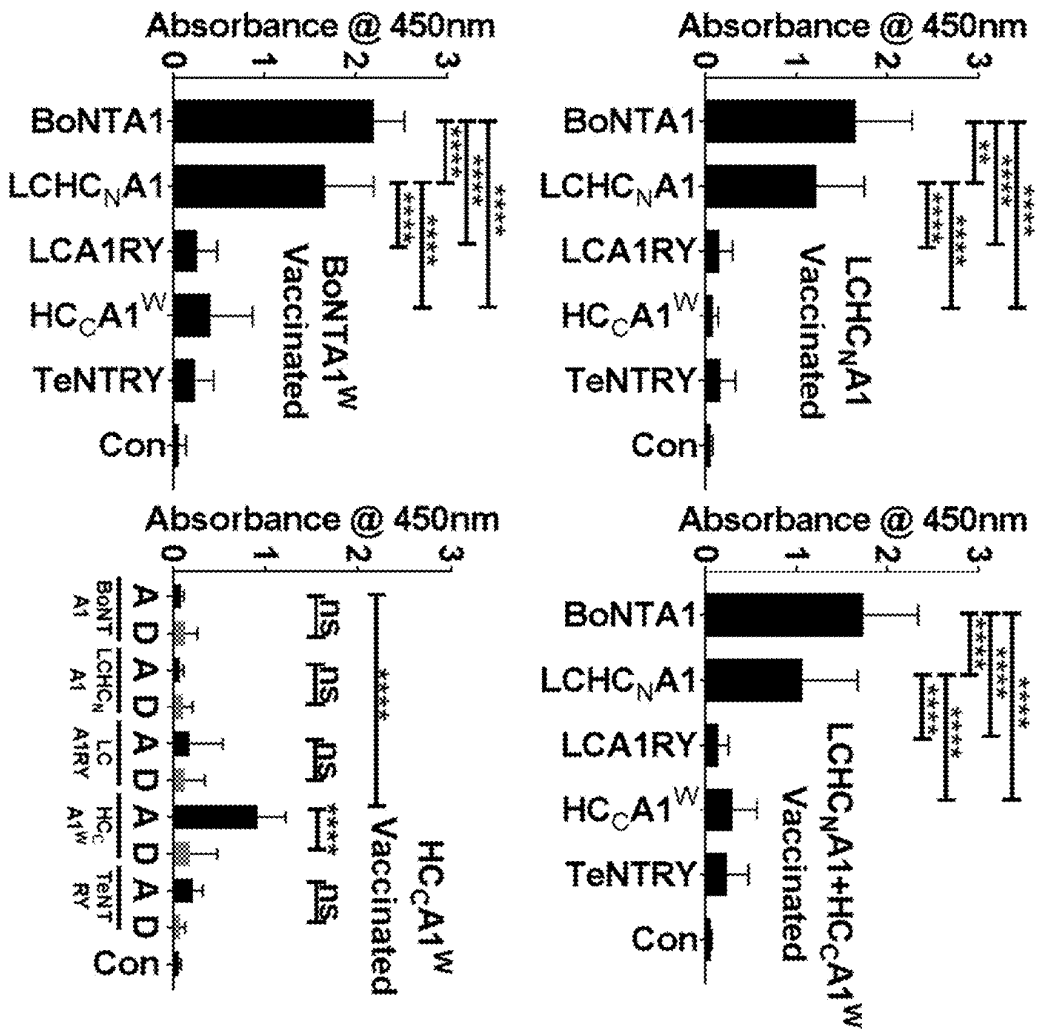


FIG. 3

FIG. 4

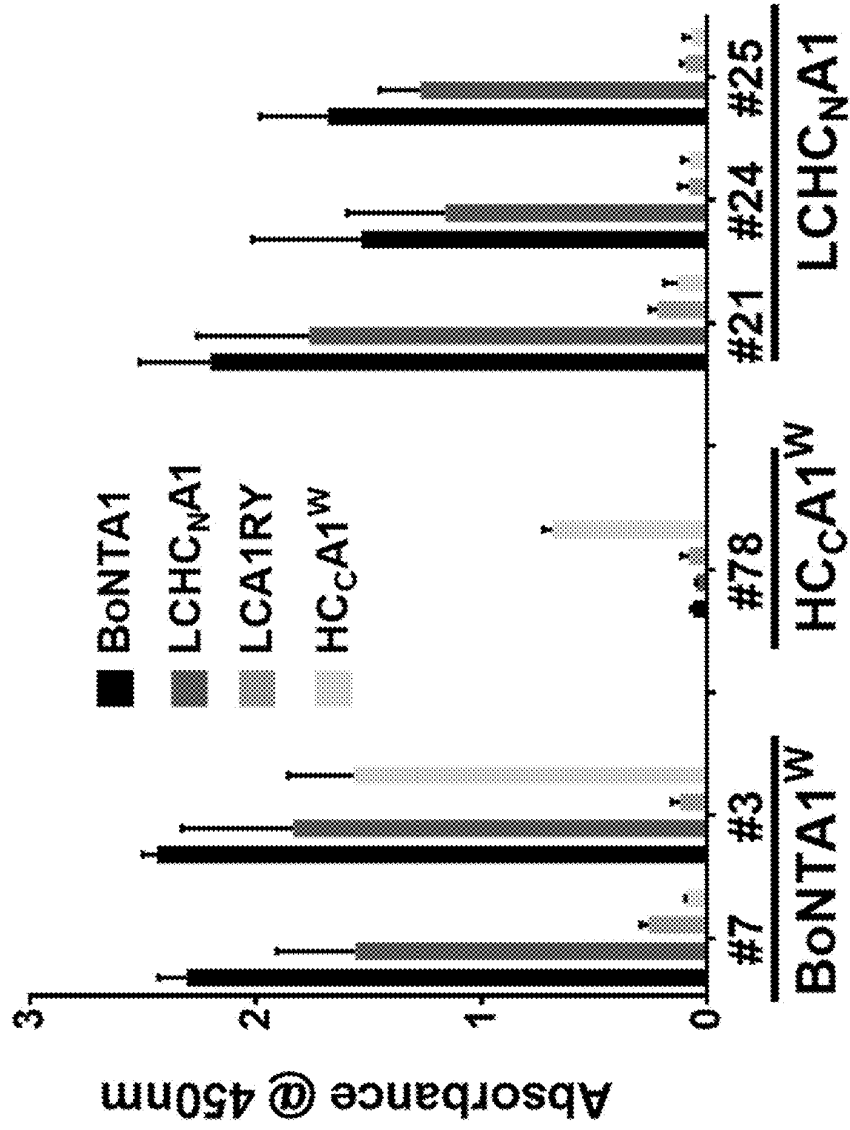
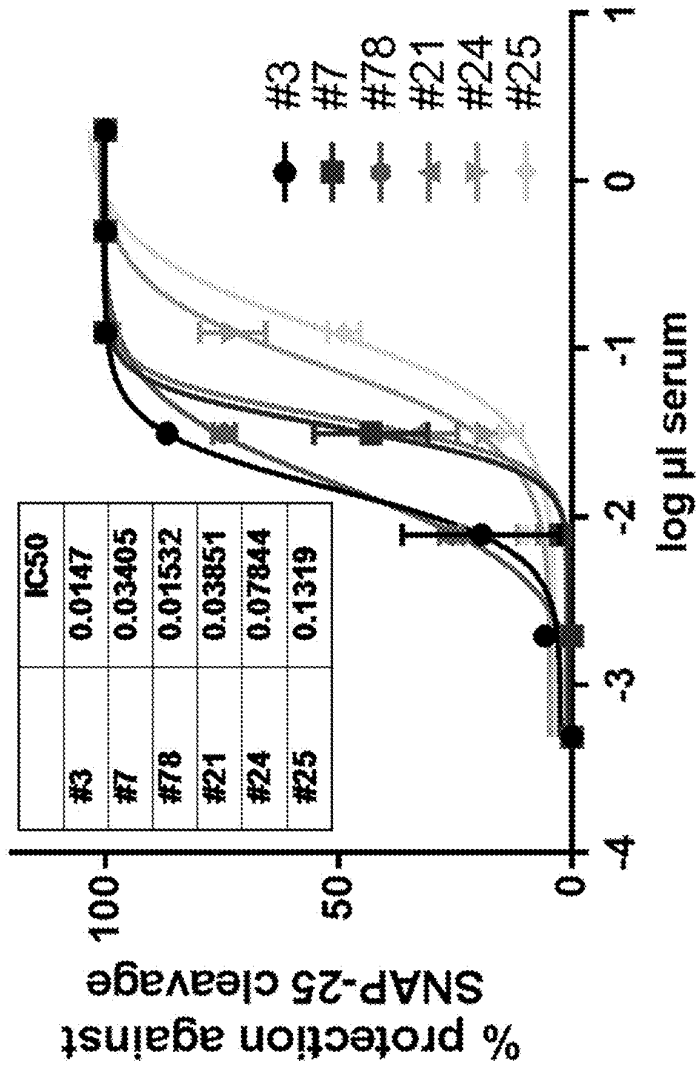
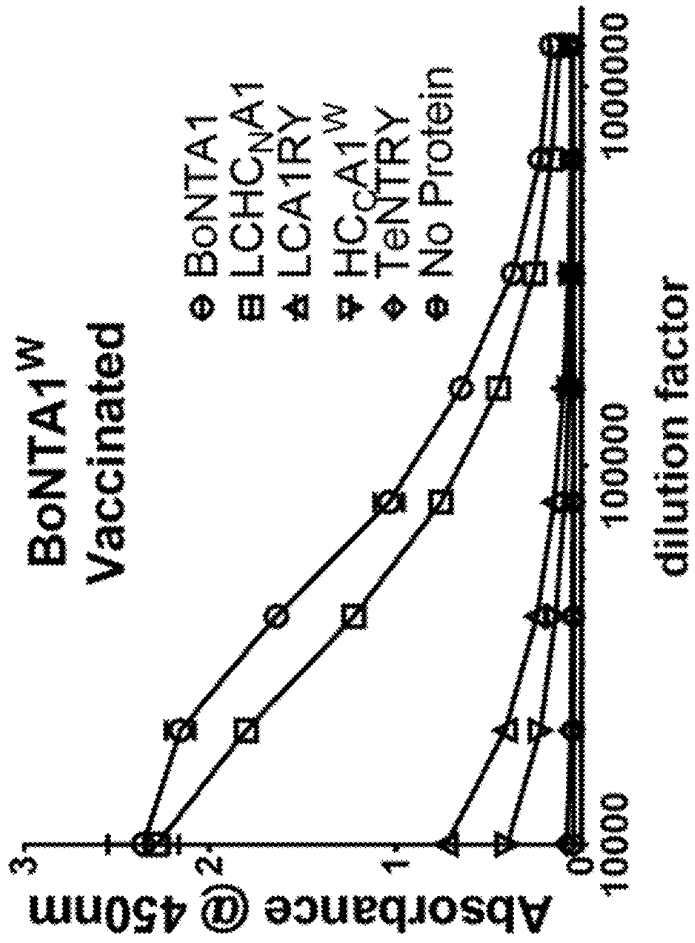


FIG. 5



FIGS 6



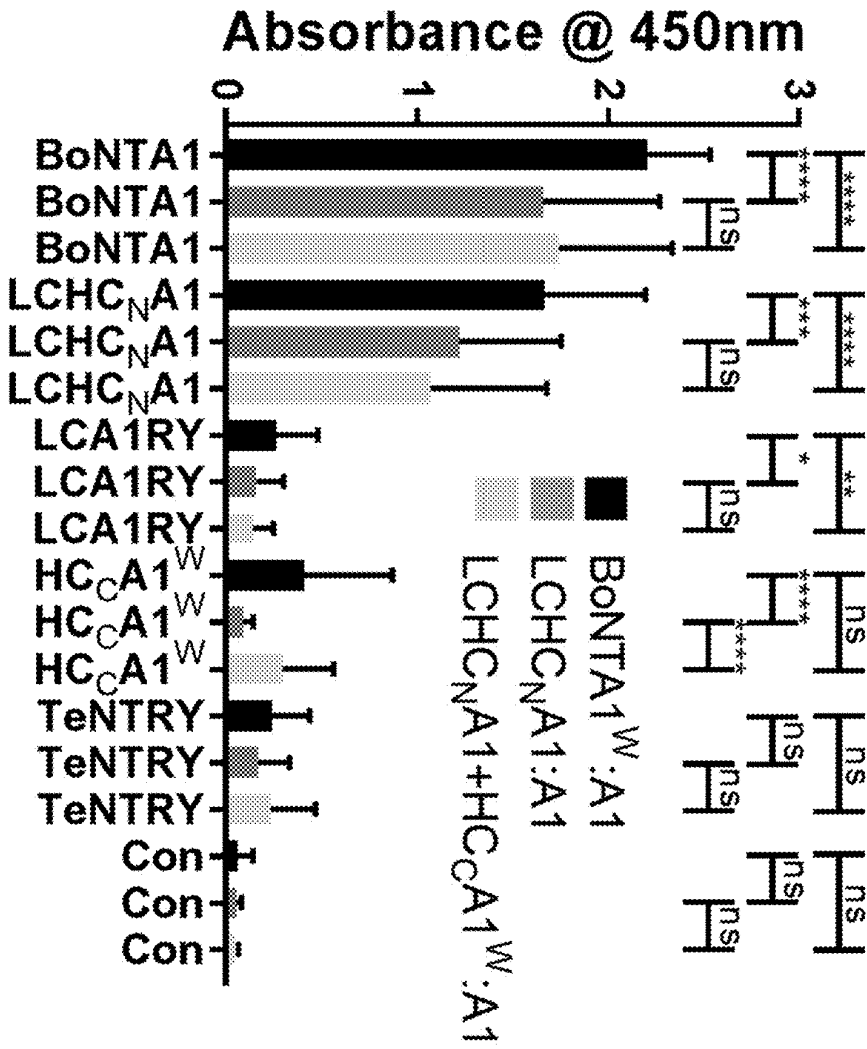


FIG. 7

FIG. 8

***Clostridium tetani* tetanus toxin (GenBank X06214.1)**

MPITINNFYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFNRKNNVAGEALLDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLMHელიHVLHGlyGMQVSSHEII
PSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNYKAIANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSN
GQYIVNEDKQILYNSIMYGFTEIELGKKFNKTRLSYFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMrvNT
NAFRNVDGSGLVSKLIGLCKKIIPPTNIRENLYNRTASLTDLGGELCIKIKNEDLTFIAEKNSFSEEPFQDEIVSYNTKNKPLNFN
YSLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKI
YSYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETTGVVLLLEYIP
EITLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKI
YSGPDKEQIADeINNLKKNLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLLEFDTQSKNILMQYIKANSKFIGITELKK
LESKINKVFSTPIPFSSYKLNDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESEVI
VHKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSIISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITF
RDLDPKFNAYLANKWWFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNTLKLDRCNNNNQYVSIDKFRIFCKALNPK
EIEKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSSKDVQLKNITDYMYLTNAPSYTNGKLNiYYRRLYNGLKfIIKRYTPN
NEIDSFVKSgDFIKLYVSYNNNEHIVGYPKDGNafNLDRIlRVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASL
GLVGTHNGQIGNDPNRDILIASNWYFNHLKDKILGCDWYFVPTDEGWTND
(SEQ ID NO:1)

FIG. 9

2M-TT (TeNTRY) (GenBank X06214.1. modified for optimized expression in *Escherichia coli*)

MPITINNFYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFRNIKNNVAGEALLDKIINAIPYLGNSYSLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLMHELIHVLHGLYGMQVSSHEII
PSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDYKAIANKLSQVTSCNDPNIDIDSYKQIYQKQYQFDKDSN
GQYIVNEDKFQILYNSIMYGFTEIELGKKFNKTA~~LS~~FFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMVNTN
AFRNVDGSGLVSKLIGLCKKIIPPTNIRENLNRTASLTDLGGELCIKIKNEDLTFIAEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIY
SYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETGTVLLLEYIPEI
TLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYS
GPDKEQIADEINNLKKNLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLLEFDTQSKNILMQYIKANSKFIGITELKLE
SKINKVFSTPIPFYSKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIV
HKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSISSMKKHLSIGSGWSVSLKGNLIWTLKDSAGEVRQITFR
DLPDKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIRDNNITLKLDRCNNNNQYVSIDKFRIFCKALNPKEI
EKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSSKDVQLKNITDYMILTNAPSYTNGLKLNIIYRRLYNGLKFIKRYTPNN
EIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNAFNLDRIILRVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASLG
LVGTHNGQIGNDPNRDILIASNWYFNHLKDKILGCDWYFVPTDEGWTND

(SEQ ID NO:2)

FIG. 10

5M-TeNT

MPITINNFYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFNRKNNVAGEALLDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLMHQLIHVLHGGLYGMQVSSHEI
IPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNDYKAIANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSN
GQYIVNEDKFQILYNSIMYGFTIEELGKKFNIKTALSFFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMVRVNTN
AFRNVGDGSGLVSKLIGLCKKIIPPTNIRENLNRTASLTDLGGELCIKIKNEDLTFAIEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIY
SYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETGTVLLLEYIPEI
TLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYS
GPDKEQIADEINNLKNKLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLEFDTQSKNILMQYIKANSKFIGITELKLE
SKINKVFSTPIPFYSKLNDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNSESSEVIV
HKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSIISSMKKHLSIGSGWSVSLKGNLIWTLKDSAGEVRQITFR
DLPDKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIRDNNITLKLDRCNNNNQYVSIDKFRIFCKALNPKEI
EKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSSKDVQLKNITDYMILTANPSYTNGLKNIYYRRLYNGLKFIKRYTPNN
EIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNFNNLDRILLVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASLG
LVGTHNGQIGNDPNRDILIASNAYFNHLKDKILGCDWYFVPTDEGWTND

(SEQ ID NO:4)

FIG. 11

6M-TeNT

MPITINNFYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFRNIKNNVAGEALLDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLMHQLIHVLHGGLYGMQVSSHEI
IPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNDYKAIANKLSQVTSCNDPNIDIDSYKQIYQOKYQFDKDSN
GQYIVNEDKFQILYNSIMYGFTIEELGKKFNIKTALSFFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMVRVNTN
AFRNVDGSGLVSKLIGLCKKIIPPTNIRENLNRTASLTDLGGELCIKIKNEDLTFAIEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIY
SYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETGTVLLLEYIPEI
TLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYS
GPD~~A~~EQIADEINNLKNKLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLEFDTQSKNILMQYIKANSKFIGITELKKE
SKINKVFSTPIPFYSKLNDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNSESSEVIV
HKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSISSMKKHSLSIGSGWSVSLKGNLIWTLKDSAGEVRQITFR
DLPDKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNTLKLDRCNNNNQYVSIDKFRIFCKALNPKEI
EKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSKDVQLKNITDYMYLTNAPSYTNGKLNIIYYRRLYNGLKFIKRYTPNN
EIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNFNNLDRILLVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASLG
LVGTHNGQIGNDPNRDILIASN~~A~~YFNHLKDKILGCDWYFVPTDEGWTND

(SEQ ID NO:5)

FIG. 12

7M-TeNT

MPITINNFYSDPVNNDTIIMMEPPACKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFNRKNNVAGEALLDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLMHQLIHVLHGGLYGMQVSSHEI
IPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDYKAIANKLSQVTSCNDPNIDIDSYKQIYQKQYQFDKDSN
GQYIVNEDKFQILYNSIMYGFTIEELGKKFNIKTALSFFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMVRVNTN
AFRNVGDGSGLVSKLIGLCKKIIPPTNIRENLNRTASLTDLGGELCIKIKNEDLTFIAEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIY
SYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETGTVLLLEYIPEI
TLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYS
GPD~~A~~EQIADEINNLKNKLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLEFDTQSKNILMQYIKANSKFIGITELKLE
SKINKVFSTPIPFYSKLNDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNSESSEVIV
HKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSISSMKKHSLSIGSGWSVSLKGNLIWTLKDSAGEVRQITFR
DLPDKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNTLKLDRCNNNNQYVSIDKFRIFCKALNPKEI
EKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSKDVQLKNITDYMILTANPSYTNGLKNIYYRRLYNGLKFIKRYTPNN
EIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNFNNLDRILLVGYNAPGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASLG
LVGTHNGQIGNDPNRDILIASN~~A~~YFNHLKDKILGCDWYFVPTDEGWTND

(SEQ ID NO:6)

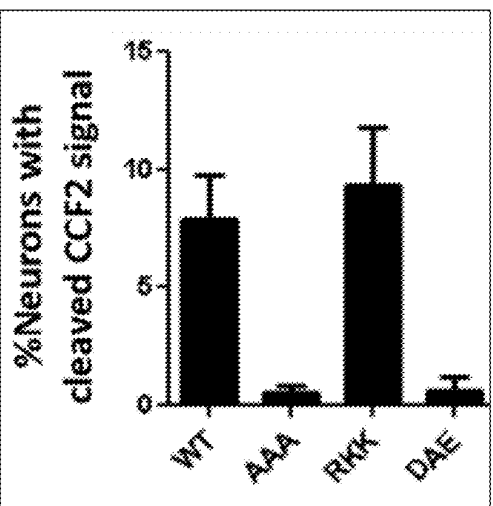
FIG. 13

8M-TeNT

MPITINNFYSDPVNNDTIIMMEPPACKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNPPSSLIEGASEYYDPNYLRTDSDKD
RFLQTMVKLFNRKNNVAGEALLDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLKMHQLIHVLHGLYGMQVSSHEI
IPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDYKAIANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSN
GQYIVNEDKFQILYNSIMYGFTIEELGKKFNIKTALSFFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMVRVNTN
AFRNVDGSGLVSKLIGLCKKIIPPTNIRENLYNRTASLTDLGGELCIKIKNEDLTFIAEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIY
SYFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETGTVLLLEYIPEI
TLPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKIIDYEYKIYS
GPD~~A~~EQIADEINNLKNKLEEKANKAMININIFMRESSRSFLVNQMINEAKKQLEFDTQSKNILMQYIKANSKFIGITELKLE
SKINKVFSTPIPFYSKLNDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIV
HKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSISSMKKHSLSIGSGWSVSLKGNLIWTLKDSAGEVRQITFR
DLPDKFNAYLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNTLKLDRCNNNNQYVSIDKFRIFCKALNPKEI
EKLYTSYLSITFLRDFWGNPLRYDTEYYLIPVASSKDVQLKNITDYMILTANPSYTNGLKNIYYRRLYNGLKFIKRYTPNN
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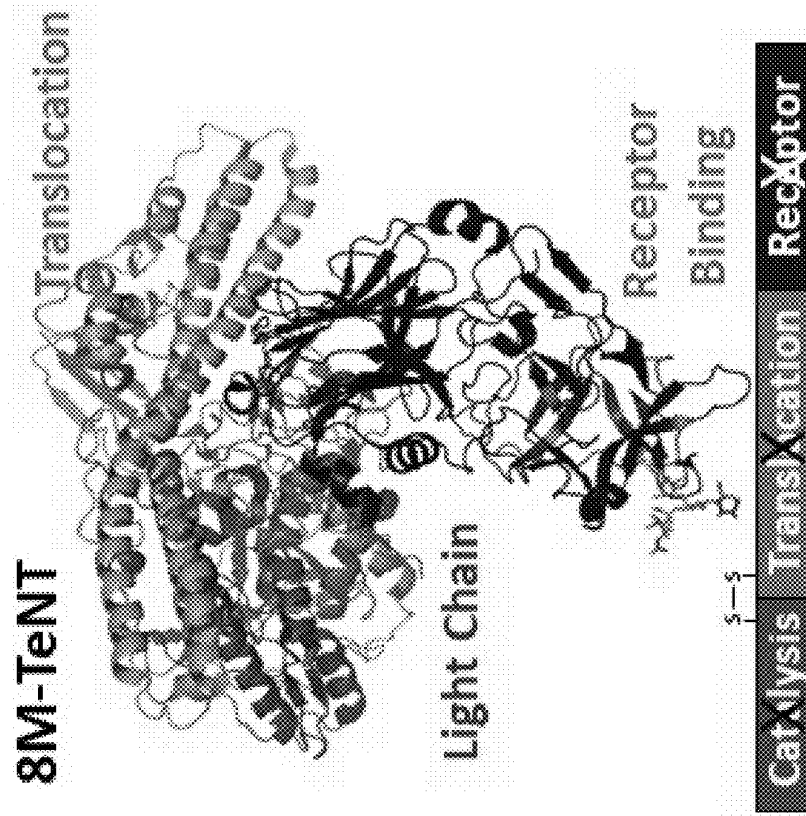
(SEQ ID NO:7)

FIG. 14



Neurotoxin	Loop sequence
TT	765-GPDKE-769
BT A1	755-EEEKN-759
BT B1	743-EKEKS-747
BT G	748-EEDKM-752
BT F	745-LDEKN-749
BT E	745-TDEKS-749
BT C	752-GSDKE-756
BT D	748-GSDKE-752

FIG. 15



1

MODIFIED CLOSTRIDIAL NEUROTOXINS AS VACCINES AND CONJUGATE VACCINE PLATFORMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage filing under 35 U.S.C. 371 of International Application No. PCT/US2018/066033, filed Dec. 17, 2018, which claims priority to U.S. Provisional Patent Application No. 62/599,444, filed on Dec. 15, 2017, both of which are incorporated by reference in their entirety as if fully set forth herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant numbers AI030162 awarded by NIH and FD-U-001418 awarded by FDA. The government has certain rights in the invention.

BACKGROUND

Botulinum neurotoxins (BoNTs), the most poisonous substances known to man, are protein toxins produced by *Clostridium botulinum* and select strains of *Clostridium butyricum* and *Clostridium baratii* (Hill and Smith, 2013; Johnson and Montecucco, 2008). BoNTs are synthesized as 150 kDa dichain proteins made up of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond. The HC is further divided into an N-terminal domain (H_N), which aids in translocation of the LC into the cell cytosol, and a C-terminal domain (H_C), which recognizes and bind to cell surface receptors on neuronal cells (Montal, 2010). Once inside the cell, the LC specifically cleaves a portion of a soluble N-ethylmaleimide sensitive-factor attachment protein receptors (SNARE), thereby inactivating neurotransmitter release (Montecucco and Schiavo, 1993, *Trends in biochemical sciences* 18, 324-327; Schiavo et al., 1995). An experimental vaccine has previously been used to protect 'at risk' populations from botulism, however, use of this chemically inactivated BoNT toxoid vaccine was discontinued due to declining potency. Also, conventional tetanus toxin fragment vaccines are not ideal because of problems with low antigenicity and immunopotency. Accordingly, there remains a need in the art for non-catalytic, non-toxic variants of tetanus and *botulinum* toxins for use as adjuvants and as conjugate vaccines.

SUMMARY OF THE DISCLOSURE

Provided herein are recombinant non-catalytic, non-toxic variant forms of tetanus toxin and uses of such variant toxins. The data described here show significantly reduced toxicity relative to native tetanus toxin and relative to previously described tetanus variants. We envision several independent engineered mutations that inactivate the intrinsic toxicity of tetanus toxin and which can be combined to produce a safe and effective vaccine, including but not limited to the elimination of catalytic activity by eliminating substrate affinity or reducing the rate of reaction, eliminating receptor binding, inhibiting translocation potential, or interfering with toxin interdomain cleavage or disulfide bond disruption, among other steps in toxin intoxication. Described herein are experiments in which we engineered toxins having mutations that reduced host receptor binding

2

along with a reduction in catalysis. These data demonstrate the potential for recombinant toxins comprising selected independent mutations that render them non-toxic and suitable for use as vaccines and conjugate vaccine without the need for chemical cross-linking to reduce toxicity.

In a first aspect, provided herein is a modified tetanus toxin polypeptide comprising a sequence having at least 95% identity to SEQ ID NO:1 and having a mutation at each of positions R372 and Y375, and further comprising a mutation at two or more positions selected from E334, K768, R1126, and W1289, where each position is numbered relative to SEQ ID NO:1, the polypeptide having reduced catalytic activity, translocation, and receptor binding compared with the toxicity and receptor binding of SEQ ID NO:1. The amino acid R at position R372 can be replaced with amino acid A, and the amino acid Y at position Y375 can be replaced with amino acid F. The mutations can comprise R372A, Y375F, E334Q, R1226L, and W1289A. The modified polypeptide can further comprise a covalently linked carbohydrate, whereby the polypeptide is a polypeptide-carbohydrate conjugate. The modified polypeptide can be encoded by SEQ ID NO:2.

In some cases, the mutations can comprise R372A, Y375F, E334Q, K768A, R1226L, and W1289A. The modified polypeptide can be encoded by SEQ ID NO:5. In some cases, the modified polypeptide can further comprise a mutation at one or both of positions L231 and Y26, where each position is numbered relative to SEQ ID NO:1. The mutations at one or both of positions L231 and Y26 comprise L231K and Y26A. The modified polypeptide can be encoded by SEQ ID NO:6 or SEQ ID NO:7.

In another aspect, provided herein is a composition comprising a modified polypeptide as described herein and a pharmaceutically acceptable carrier.

In a further aspect, provided herein is a method of reducing the risk of a subject developing tetanus by inducing an immune response through administering to the subject a therapeutically effective amount of a modified polypeptide as described herein. In some cases, the modified polypeptide is used as an adjuvant. In some cases, the modified polypeptide is used as a vaccine.

The foregoing and other aspects and advantages of the invention will appear from the following description. In the description, reference is made to the accompanying drawings which form a part hereof, and in which there are shown, by way of illustration, preferred embodiments of the invention. Such embodiments do not necessarily represent the full scope of the invention, however, and reference is made therefore to the claims and herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates recombinant proteins used to assess the host immune response to vaccination. (Upper panel) Schematic of BoNT-derivatives used in this study are shown. Where indicated, two epitopes (His₆ and Strep) were used for protein purification. 3XFLAG (3XF) and two sequential hemagglutinin (2HA) epitopes were included for cellular studies. Domain junctions were defined, using the crystal structure of BoNT/A1 (PDB:3BTA). Single amino acid designation above each schematic indicates the introduction of amino acid substitutions introduced to reduce catalysis (LC) or receptor binding (H_C). Note, single chain BoNT and LCHC_N were used for vaccination. (Lower panel) Four μg of the indicated proteins were subjected to SDS-PAGE and Coomassie blue staining. Lanes: 1, M-BoNT/A1; 2,

3

M-BoNT/A1 trypsin nicked and reduced; 3, M-LCHC_N/A1; 4, M-LCHC_N/A1 trypsin nicked and reduced; 5, LC/A1^{RY}; 6, HC_C/A1^W; and 7, TeNT^{RY}. Migration of molecular weight marker proteins (kDa) are shown in left lane. Note in lane 2 nicked HC runs at ~80 kDa, which was shown in other experiments to be due to cleavage of the belt region of HC by trypsin.

FIG. 2 is an ELISA of sera from mice vaccinated with M-BoNT/A1 or M-BoNT/A1¹ and challenge by native BoNT/A2, a heterologous subtype. Mice were vaccinated with M-BoNT/A1 (upper panel) or M-BoNT/A1¹ (lower panel) and sera collected prior to a 10⁶ LD50 native BoNT/A2 challenge. Mice surviving (A) or non-surviving (D) challenge are indicated. ELISAs were performed measuring antibody titers of sera (1:20,000 dilution) for M-BoNT/A1; M-LCHC_N/A1; LC/A1^{RY}; HC_C/A1^W; TeNT^{RY}; and no protein control (Con). Bound mouse antibodies were detected with goat α-mouse IgG-HRP (1:20,000 dilution), using TMB reagent. Reactions were stopped with dilute H₂SO₄ and read @ 450 nm. Data are presented as the average of 5 mice (A) and 4 mice (D) following M-BoNT/A1 vaccination and 3 mice (A) and 6 mice (D) following M-BoNT/A1^W vaccination from two independent experiments performed in duplicate with standard deviation indicated. Statistical analyses were performed as described herein: P, 0.05=*

FIG. 3 is an ELISA of sera from mice vaccinated with BoNT derivatives and challenged with native BoNT/A1. Mice were vaccinated with M-BoNT/A1^W (0.3 μg), M-LCHC_N/A1 (0.2 μg), M-LCHC_N/A1 (0.2 μg)+HC_C/A1^W (0.1 μg), or HC_C/A1^W (0.3 μg). Sera were obtained prior to BoNT challenge. ELISAs determining antibody titers of sera (1:30,000 dilution) for M-BoNT/A1; M-LCHC_N/A1; LC/A1^{RY}; HC_C/A1^W; TeNT^{RY}; and no protein control (Con). Bound mouse antibodies were detected with goat α-mouse IgG-HRP (1:20,000 dilution), using TMB reagent. Reactions were stopped with dilute H₂SO₄ and read @ 450 nm. Data are presented as the average of 10 independent sera of mice surviving BoNT/A1 challenge from Experiment 3 in Table 1 analyzed in two independent experiments performed in duplicate with standard deviation indicated; except for mice vaccinated with HC_C/A1^W where the data are from 7 survivors (A) or 3 non-survivors (D) of native BoNT/A1 challenge. Variance in the range of titers was due to the varied antibody titers among individual mice, not to variance in the ELISA replicates. Statistical analyses were performed as described in Methods Section: P<0.05=*, 0.01=**, 0.001=***, and 0.0001=****.

FIG. 4 is an ELISA of serum from individual mice vaccinated with M-BoNT derivatives and surviving challenge by native BoNT/A1. Sera obtained prior to BoNT challenge from individual mice vaccinated with BoNT/A1^W (#7 and #3), HC_C/A1^W (#78) and LCHC_N/A1 (#21, #24, and #25) surviving native BoNT/A1 challenge were analyzed by ELISA (1:30,000 dilution) using M-BoNT/A1; M-LCHC_N/A1; LC/A1^{RY}; HC_C/A1^W as antigens. Bound mouse antibodies were detected with goat α-mouse IgG-HRP (1:20,000 dilution), using TMB reagent. Reactions were stopped with dilute H₂SO₄ and read @ 450 nm. Data presented are the average of two independent experiments each performed duplicate with standard deviation indicated.

FIG. 5 demonstrates serum neutralization of native BoNT/A1 cleavage of SNAP25 in human induced pluripotent stem cells (hiPSCs). Sera obtained prior to BoNT challenge from individual mice vaccinated with M-BoNT/A1^W (#7 and #3), HC_C/A1^W (#78) and M-LCHC_N/A1 (#21, #24, and #25) surviving BoNT/A1 challenge were analyzed for their capacity to neutralize native BoNT/A1. Human

4

induced pluripotent stem cell (hiPSC)-derived neurons were seeded into poly-L-ornithine and Matrigel coated plates at a density of 35,000-40,000 cells per well and maintained in iCell Neurons culture media for 7 days prior to the neutralization assay. To detect neutralizing antibodies in the mouse sera, 2 pM of native BoNT/A1 was combined with serial dilutions of sterile filtered sera and in culture media and incubated for 1 hour at 37° C. A 'no-antibody' buffer was used as a control. Fifty μl of each antibody-toxin mixture was added per well of hiPSC derived neurons in at least duplicates and cells were incubated for 24 h at 37° C., 5% CO₂. The toxin/antibody was aspirated from the cells, and cell lysates were subjected to PAGE followed by analyzed by Western blot for SNAP-25 cleavage (Pellett et al., 2007; Pellett et al., 2010). Cleaved versus noncleaved SNAP-25 was quantified by densitometry and the % of protection was determined by comparison to the 'no-antibody' control. IC₅₀ values were estimated using GraphPad Prism 6 software and a nonlinear regression, variable slope, four parameters.

FIG. 6 is a representative ELISA of serum from a mouse vaccinated with M-BoNT/A1^W that survived challenge by BoNT/A1. Serum from a BoNT/A1^W vaccination of a mouse surviving BoNT/A1 challenge was analyzed by ELISA as described in the Methods Section, using the indicated antigens. Values are the average of a representative determination performed in duplicate with standard deviation indicated.

FIG. 7 demonstrates that M-BoNT/A1^W is more immunogenic than M-LCHC_N/A1, or LCHC_N/A1+HC_C/A1^W. Sera from individual mice vaccinated with M-BoNT/A1 (0.3 μg), M-LCHC_N/A1^W (0.2 μg), or M-LCHC_N/A1 (0.2 μg)+HC_C/A1^W (0.1 μg) surviving challenged with 10⁶ LD₅₀BoNT/A1 were analyzed by ELISA for antibodies to M-BoNT/A1, M-LCHC_N/A1, LC/A1^{RY}, HC_C/A1^W, TeNT^{RY}, or no protein (Con). Data are presented as the average of 10 independent sera of mice surviving BoNT/A1 challenge from Experiment 3 in Table 1 analyzed in two independent experiments performed in duplicate with standard deviation indicated. Statistical analyses were performed as described in Methods Section: P<0.05=*, 0.01=**, 0.001=***, and 0.0001=****.

FIG. 8 is an amino acid sequence encoding wild-type *Clostridium tetani* tetanus toxin (SEQ ID NO:1).

FIG. 9 is an amino acid sequence encoding 2M-TT (SEQ ID NO:2).

FIG. 10 is an amino acid sequence encoding 5M-TeNT (SEQ ID NO:4).

FIG. 11 is an amino acid sequence encoding 6M-TeNT (SEQ ID NO:5).

FIG. 12 is an amino acid sequence encoding 7M-TeNT (SEQ ID NO:6).

FIG. 13 is an amino acid sequence encoding 8M-TeNT (SEQ ID NO:7).

FIG. 14 demonstrates that K768 mediates light chain translocation in tetanus toxin. (left panel) TT⁷⁶⁷DKE (WT), TT⁷⁶⁷AAA, TT⁷⁶⁷RKK, or TT⁷⁶⁷DAE were incubated with neurons and assayed for translocation as reporter (β-lactamase) CCF2 cleavage. (right panel) Alignment of TT and BTs at ⁷⁶⁷DKE. Note the conserved lysine (K) among toxins.

FIG. 15 is a crystal structure of TeNTRY PDB:5n0b. Four TT functions were inactivated: Light Chain E234Q, R373A, Y376F (Zn⁺⁺ binding), L231K (VAMP-2 cleavage) and Y26A (VAMP-2 binding), K768A (LC translocation), and R1226L and W1289A (receptor binding).

DETAILED DESCRIPTION OF THE DISCLOSURE

The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated

that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

The methods and compositions described herein are based at least in part on the inventor's development of genetically engineered toxins that are both non-catalytic and incapable of neuronal cell binding. As described in the paragraphs and examples that follow, tetanus toxins and *botulinum* toxins (BoNT) having engineered defects that hinder catalysis and receptor binding provide a platform for vaccine development for toxin-mediated diseases. For example, no toxicity was detected with modified tetanus toxin and BoNT rendered non-catalytic and incapable of receptor binding via engineered mutations. Moreover, such modified toxins were suitable as vaccines to protect against *botulinum* neurotoxin challenge.

Compositions

Preferably, genetically modified toxins of the present disclosure comprise genetic modifications at multiple targets relative to a wild-type toxin, where such modifications eliminate residual toxicity and enhance safety, which will be expanded upon below. Although BoNT and tetanus toxin act on different substrates and receptors, the inventors determined that immune cells take up a non-catalytic, non-receptor binding form of BoNT (referred to herein as "M-BoNT^{WT}") and mount a similar neutralizing immune response as observed with a non-catalytic BoNT ("M-BoNT"). Based on this observation, additional independent sites of mutation were determined to engineer other non-catalytic, non-receptor toxin variants. When modified as described herein, the resulting engineered non-catalytic, non-receptor binding toxins are suitable for use as vaccine and conjugate vaccine platforms.

Without being bound to any particular theory or mode of action, mutations engineered at independent sites in tetanus toxin and *botulinum* toxin render the toxin proteins unable to express toxicity via independent mechanisms, thus providing a fail-safe against inadvertent genetic reversion to toxicity. Such properties are advantageous for use of variant toxins as vaccines against tetanus and botulism and as conjugate vaccine platforms.

Accordingly, provided herein are recombinantly inactivated bacterial toxins that are irreversibly non-toxic, more potent, and easier to produce and manipulate than current chemically inactivated toxoid and, thus, provide improved vaccines and conjugate vaccine carriers. In a first aspect, provided herein are isolated preparations of recombinant non-catalytic, non-toxic modified forms of bacterial protein toxins (e.g., tetanus toxin and *botulinum* neurotoxin), where the modified toxins are full length toxins comprising at least four amino acid substitutions that render the proteins incapable of expressing toxicity by independent mechanisms. By "preparation" we mean any concentration of the toxin polypeptide that is enhanced or purified relative to its natural occurrence. Preferably, the preparation is substantially pure or is combined with other ingredients into a pharmaceutical preparation. In some cases, a preparation of the present invention may include one or more adjuvants or carriers that might be coupled to the toxin polypeptide sequence help to stimulate the immune system. In other cases, the preparation itself has adjuvant activity and is effective to boost an immune response to a conjugated antigen or co-administered antigen.

As used herein, "toxin" refers to a noxious or poisonous substance (e.g., a cytotoxin) that is formed or elaborated either as an integral part of a cell or tissue (endotoxin), as an intracellular or extracellular product (exotoxin), or as a

combination thereof, during the metabolism and growth of certain microorganisms. As used herein, the term "modified toxin" refers to a non-catalytic, non-toxic variant form of a toxin, wherein the toxin is rendered non-catalytic and non-toxic by genetically engineered (e.g., non-naturally occurring, man-made) modifications to the amino acid sequence of the polypeptide toxin. In exemplary embodiments, modified toxins are genetically engineered or otherwise modified variants of a toxin produced by a bacterium of the genus *Clostridium* (e.g., *C. difficile*, *C. novyi*, *C. sordellii*, *C. perfringens*, *C. tetani*, and *C. botulinum*). The toxins may be recombinant, synthetic, part of a fusion protein (which includes, e.g., an antigen, or a polypeptide (e.g., His₆) which facilitates purification of the fusion protein), covalently conjugated to an antigen, and/or chemically cross-linked to an antigen. In some cases, non-catalytic, non-toxic forms of toxins are referred to as toxoids. Toxoids lack toxicity but retain their antigenicity and their immunizing capacity.

As used herein, the term "reduced toxicity" means that, relative to a first composition comprising a particular protein active ingredient (e.g., wild-type TT), a second composition comprising a modified version of a particular protein active ingredient can be administered to a mammal at a dose level which is the same or greater than what is a fatal for the first composition but without death resulting to the mammal. Reduced toxicity encompasses partially or completely eliminated toxicity as detectable by methods known to those who practice in the art. In addition, reduced toxicity encompasses reduced systemic toxicity (i.e., upon intravenous administration) or reduced toxicity upon intramuscular administration.

In certain embodiments, the preparation comprises a modified tetanus toxin. Typically, a tetanus toxin modified as described herein exhibits one or more altered properties as compared to the wild-type tetanus toxin polypeptide shown in SEQ ID NO:1, for example, significantly decreased catalytic activity and receptor binding activity. In some embodiments, the modified tetanus toxins described herein are at least 1,000,000 times less toxic than wild-type tetanus toxin.

TABLE 1

Exemplary toxins M-BoNT/A1 ^{WT} and 5M-TeNT comprising multiple functionally independent mutations as a vaccine and conjugate vaccine		
Residues mutated (function inhibited)	M-BoNT/A1 ^{WT}	5M-TeNT
E (catalysis)	E224A	E334Q
R (catalysis)	R363A	R372A
Y (catalysis)	Y366F	Y375F
R (receptor binding)	Wild-type	R1226L
W (receptor binding)	W1266A	W1289A

In certain embodiments, the preparation comprises a modified tetanus toxin having mutations at amino acid residues 372 and 375, and further having a mutation at one or more of residues 334, 1226, and 1289, where the residue positions are numbered relative to the full-length wild-type tetanus neurotoxin (*Clostridium tetani* CN3911; GenBank accession no. X06214) set forth as SEQ ID NO:1. In certain embodiments, the amino acid mutations at residues 372 and 375 are R372A and Y375F, and the modified toxin further comprises at least one mutation selected from E334Q, R1226L, and W1289A. In some cases, the modified toxin comprises five mutations (R372A, Y375F, E334Q, R1226L, and W1289A) numbered relative to SEQ ID NO:1 and is

referred to herein as “5M-TeNT” or “5M-TT.” See Table 1. In some cases, 5M-TeNT is encoded by the amino acid sequence set forth as SEQ ID NO:4.

In some embodiments, the tetanus toxin has a modified translocation domain. For example, the lysine (K) residue at position 768 is located within a loop that connects two long alpha helices. Mutation of this single amino acid to an alanine (A) inactivates or blocks light chain translocation. In some cases, the K768A mutation is added to 5M-TT modified toxin to produce 6M-TT, whereby the resulting modified tetanus toxins comprises independent mutations at six positions (see Tables 2 and 3). In some cases, the modified toxin comprises six mutations (R372A, Y375F, E334Q, K768A, R1226L, and W1289A) numbered relative to SEQ ID NO:1 and is referred to herein as “6M-TeNT” or “6M-TT.” In some cases, 6M-TeNT is encoded by the amino acid sequence set forth as SEQ ID NO:5. Without being bound by any particular mechanism or theory, vaccine potency of 6M-TT is expected to be higher than 5M-TT but should have a lower rate of reversion than 5M-TT. The addition of a mutation at one or more of D767, K768, or E769A to 5M-TT

prizes independent mutations at seven positions (Table 3). In some cases, the modified toxin comprises eight independent mutations (R372A, Y375F, E334Q, R1226L, W1289A, K768A, and L231K) numbered relative to SEQ ID NO:1 and is referred to herein as “7M-TeNT” or “7M-TT.” In some cases, 7M-TeNT is encoded by the amino acid sequence set forth as SEQ ID NO:6.

In some embodiments, the tetanus toxin has been modified to inhibit VAMP-2 binding. For example, mutation of the tyrosine (Y) residue at position 26 (for example, mutation of the tyrosine to an alanine (A)) inactivates VAMP-2 binding capacity of the toxin. In some cases, the Y26A mutation is added to 7M-TT modified toxin to produce 8M-TT, whereby the resulting modified tetanus toxins comprises independent mutations at eight positions (Table 3). In some cases, the modified toxin comprises eight independent mutations (R372A, Y375F, E334Q, R1226L, W1289A, K768A, L231K, and Y26A) numbered relative to SEQ ID NO:1 and is referred to herein as “8M-TeNT” or “8M-TT.” In some cases, 8M-TeNT is encoded by the amino acid sequence set forth as SEQ ID NO:7.

TABLE 3

Exemplary mutations for inactivating multiple independent TT functions				
	Zn++ binding	Substrate Binding & catalysis	Light Chain translocation	Ganglioside receptor binding
	E234Q, R372A, Y375F (TT(R372A, Y375F, 2M-TT) is 125,000-fold less toxic than native TT ⁴²)	Y26A, L231K	K768A Inhibits LC translocation (preliminary data)	R1226L, W1289A This mutation is ~800-fold less toxic than TT WT
2MTT	R372A, Y375F			
5M-TT	E234Q, R372A, Y375F			R1226L, W1289A
6M-TT	E234Q, R372A, Y375F		K768A (or D767A or E769A)	R1226L, W1289A
7M-TT	E234Q, R372A, Y375F	L231K	K768A (or D767A or E769A)	R1226L, W1289A
8M-TT	E234Q, R372A, Y375F	Y26A, L231K	K768A (or D767A or E769A)	R1226L, W1289A

will yield more complete inactivation of the genetically engineered vaccine by inactivating a function of the translocation domain in addition to disrupted functionality of the catalytic and receptor binding domains.

TABLE 2

Exemplary toxins 6M-TeNT comprising multiple functionally independent mutations as a vaccine and conjugate vaccine		
Residues mutated (function inhibited)	M-BoNT/A1 ^W	6M-TeNT (“6M-TT”)
E (catalysis)	E224A	E334Q
R (catalysis)	R363A	R372A
Y (catalysis)	Y366F	Y375F
R (receptor binding)	Wild-type	R1226L
W (receptor binding)	W1266A	W1289A
K (light chain translocation)	Wild-type	K768A (or D767A or E769A)

In some embodiments, the tetanus toxin has been modified to inhibit VAMP-2 cleavage. For example, mutation of the leucine residue at position 231 (for example, mutation of the leucine to a lysine (K)) inactivates the toxin’s catalytic activity for VAMP-2 cleavage. In some cases, the L231K mutation is added to 6M-TT modified toxin to produce 7M-TT, whereby the resulting modified tetanus toxins com-

In some cases, the modified tetanus toxin comprises other amino acid substitutions at residue positions 372, 275, 334, 768, 1226, 1289, 231, and/or 26. For example, amino acids that may substitute for the listed amino acids include substitutions that reverse the charge or hydrophobicity reversal of the original residue, conservative amino acid substitutions, and substitutions that delete the original residue.

As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative amino acid 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.

Conservative amino acid substitutions are art recognized substitutions of one amino acid for another amino acid having similar characteristics. Conservative amino acid substitutions may be achieved by modifying a nucleotide sequence to introduce a nucleotide change that will encode the conservative substitution. For example, each amino acid may be described as having one or more of the following characteristics: electropositive, electronegative, aliphatic, aromatic, polar, hydrophobic and hydrophilic. Conservative substitutions include substitution among amino acids within

each group. Acidic amino acids include aspartate, glutamate. Basic amino acids include histidine, lysine, arginine; aliphatic amino acids include isoleucine, leucine and valine. Aromatic amino acids include phenylalanine, glycine, tyrosine and tryptophan. Polar amino acids include aspartate, glutamate, histidine, lysine, asparagine, glutamine, arginine, serine, threonine and tyrosine. Hydrophobic amino acids include alanine, cysteine, phenylalanine, glycine, isoleucine, leucine, methionine, proline, valine and tryptophan. Amino acids may also be described in terms of relative size, where alanine, cysteine, aspartate, glycine, asparagine, proline, threonine, serine, valine, are considered to be small.

In some cases, non-conservative substitutions are possibly provided if these substitutions 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 and do not restore toxicity.

In certain embodiments, the modified tetanus toxin comprises mutations at amino acid residues 372 and 375, and further comprises a mutation at one or more of residues 334, 1226, and 1289, where the modified toxin is conjugated or coupled to another peptide, as described below, for appropriate therapeutic methods. Advantageously, modified tetanus toxins of this disclosure do not require detoxification with formalin for use as a vaccine or adjuvant. In some cases, small quantities of formalin (~0.04%) or another fixative or stabilizing reagent (e.g., formalin, glutaraldehyde, β -propiolactone and the like) are added to the modified tetanus toxin as a stabilizing agent, but such quantities are smaller (e.g., smaller by an order of magnitude) than those generally used (~0.4%) to detoxify wild-type tetanus toxin (or tetanus toxin not modified as described herein) to form "tetanus toxoid."

In certain embodiments, modified toxins described herein further comprise molecules such as carbohydrates, protein or peptide (e.g., antigens), and chemical moieties. In particular, provided herein are recombinant non-catalytic, non-toxic variant toxin forms (e.g., modified tetanus toxin and modified *botulinum* neurotoxin) further modified to comprise a conjugated or chemically linked (e.g., cross-linked) carbohydrate. In this manner, the modified toxins conjugated to carbohydrates provide a platform for use as T-cell dependent immunogens. In some cases, other molecules or moieties (e.g., antigens) can be further linked to the cross-linked moiety as "cargo."

In some cases, a modified, carbohydrate-conjugated tetanus toxin comprises five mutations (R372A, Y375F, E334Q, R1226L, and W1289A) numbered relative to SEQ ID NO:1 and is referred to herein as "TeNT(CB)." In other cases, a modified, carbohydrate-conjugated BoNT toxin comprises four mutations (E224A, R363A, Y366F, and W1266A) numbered relative to UniProtKB/Swiss-Prot: P10845.4 and is referred to herein as "BoNT(CB)." In some cases, carbohydrates are conjugated to the modified toxin by chemical cross-linking. Common chemical reactions for covalently linking polysaccharides to polypeptides such as toxin include, without limitation, reductive amination, cyanation conjugation, and carbodiimide reactions. In other cases, carbohydrate-toxin conjugates are prepared by other synthetic schemes such as the scheme described by Chu et al., 1983. *Infect and Immun.* 40(1):245-256.

The term "carbohydrate" as used herein is intended to include polysaccharides, oligosaccharides and other carbohydrate polymers, including monomeric sugars. Typically, polysaccharides have from about 10 to up to 2,000 or more repeating units, and preferably from about 100 to 1900 repeating unit. Oligosaccharides typically about from about

2 to 10 repeating units to about 15, 20, 25, 30, or 35 to about 40 or 45 repeating units. In some cases, carbohydrates suitable for conjugation to modified toxins provided herein include, without limitation, polysaccharides that have carboxyl groups. In such cases, the polysaccharide having carboxyl groups can be conjugated through a thiol derivative of said carboxyl groups to the modified toxin.

The terms "polypeptide," "peptide," and "protein," as used herein, refer to a polymer comprising amino acid residues predominantly bound together by covalent amide bonds. By the term "protein," we mean to encompass all the above definitions. The terms apply to amino acid polymers in which one or more amino acid residue may be an artificial chemical mimetic of a naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms may encompass amino acid chains of any length, including full length proteins, wherein the amino acids are linked by covalent peptide bonds. The protein or peptide may be isolated from a native organism, produced by recombinant techniques, or produced by synthetic production techniques known to one skilled in the art.

In some cases, spacer moieties are employed as spacer arm bridges between the modified toxin and linked molecule. The spacer moiety can be any of a wide variety of molecular structures including, without limitation, dextran, polyglutamic acid, and oligopeptides.

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 at 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.

Polypeptides and nucleic acids of the invention may be prepared synthetically using conventional synthesizers. 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.

In another embodiment, the present invention is a nucleic acid sequence which codes for the modified toxin preparations and other nucleic acid sequence which hybridize to a nucleic molecule consisting of the above-described nucleotide sequences under high stringency conditions. In a particular-embodiment provided herein are DNA sequences encoding the modified tetanus toxin having mutations at amino acid residues 372 and 375, and further comprises a mutation at one or more of residues 334, 1226, and 1289 or

the modified catalytic domain described herein. In some cases, the nucleic acid sequence encoding a modified tetanus toxin is set forth as SEQ ID NO:3.

The term "stringent conditions" as used herein refers to parameters with which the art is familiar. For example, nucleic acid hybridization parameters may be found in references which compile such methods, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, high stringency conditions as used herein, refers to hybridization at 65° C. in hybridization buffer (3.5×SSC, 0.02% Ficoll, 0.02% Polyvinylpyrrolidone, 0.02% Bovine Serum Albumin, 25 mM NaH₂PO₄ (pH 7), 0.5% SDS, 2 mM EDTA). SSC is 0.15M Sodium Chloride/0.015M Sodium Citrate, pH 7; SDS is Sodium Dodecyl Sulphate; and EDTA is Ethylene diaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2×SSC at room temperature and then at 0.1-0.5×SSC/0.1×SDS at temperatures up to 68° C., e.g., 55° C., 60° C., 65° C. or 68° C. Alternatively, high stringency hybridization may be performed using a commercially available hybridization buffer, such as ExpressHyb™ buffer (Clontech) using hybridization and washing conditions described by the manufacturer.

It will also be understood that the invention embraces the use of the sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g. *E. coli*), or eukaryotic (e.g., dendritic cells, CHO cells, COS cells, yeast expression systems, recombinant baculovirus expression in insect cells). The expression vectors require that the pertinent sequence, i.e., those described supra, be operably linked to a promoter.

In another aspect, provided herein is an immunogenic composition comprising a modified toxin as described herein which, upon introduction into a host, will confer immunity to that host, in the event the host is subsequently challenged by the same microorganism (e.g., tetanus bacillus), which produced the protein(s). In preferred embodiments, the immunogenic composition is a vaccine comprising a modified toxin as described herein and further comprising an excipient and/or diluent appropriate where composition is to be administered to a subject in need of vaccination against developing disease caused by tetanus bacilli (*Clostridium tetani*) or *Clostridium botulinum*, or their purified toxins.

The term "vaccine," as used herein, refers to a composition that includes an antigen. Vaccine may also include a biological preparation that improves immunity to a particular disease. A vaccine may typically contain an agent, referred to as an antigen, that resembles a disease-causing microorganism, and the agent may often be made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The antigen may stimulate the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters. Similarly, the modified toxin preparations, combined with vaccines against other pathogens, could "boost" the immune responses to the pathogen of interest, by acting themselves as vaccine adjuvants. Adjuvants can be classified according to their physicochemical properties or mechanisms of action. The two major classes of adjuvants include compounds that directly act on the immune system such as bacterial toxins that stimulate

immune responses, and molecules able to facilitate the presentation of antigens in a controlled manner and behaving as a carrier.

Selection of appropriate vaccine components is within the routine capability of the skilled person. 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 ethyloleate. 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.

In some cases, the preparation described herein may include purified modified toxins, including preparation comprising partial toxin complexes. In some embodiments, the preparations may further include stabilizers that are known to stabilize the modified BoNT and tetanus toxin proteins. Suitable stabilizers are known in the art, and include, but are not limited to, for example, human or bovine serum albumin, gelatin, recombinant albumin as described in US Publication US2005/0238663 (the contents of which are incorporated by reference in its entirety) among others.

The terms "subject" and "patient" are used interchangeably and refer to any animal (e.g., a mammal), including, but not limited to, humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

A preparation of the present invention can be administered in a therapeutically effective amount. The terms "effective amount" or "therapeutically effective amount" refer to an amount of an antigen or vaccine that would induce an immune response in a subject receiving the antigen or vaccine which is adequate to prevent signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a pathogen, such as a virus or a bacterium. Humoral immunity or cell mediated immunity or both humoral and cell mediated immunity may be induced. The immunogenic response of an animal to a vaccine may be evaluated, e.g., indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild-type strain. The protective immunity conferred by a vaccine may be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The amount of a vaccine that is therapeutically effective may vary depending on the particular preparation used, or the condition of the subject, and may be determined by a physician.

A preparation of the present invention can be administered in a therapeutically effective amount depending on the type of treatment necessary. Methods of determining suitable dosage or dosage ranges for individual treatment are known to those in the art. For methods provided herein, a preparation of the present invention can be administered by any means that achieves the intended purpose or is deemed appropriate by those skilled in the art. In an exemplary embodiment, a modified toxin preparation is administered either as a single dose or, when appropriate, as continuous administration using, for instance, a mini pump system. In

some cases, a modified toxin preparation is provided as a liquid dosage form or as a lyophilized dosage form that is, for example, reconstituted prior to administration.

The term “protected,” as used herein, refers to immunization of a patient against a disease or condition. The immunization may be caused by administering a vaccine comprising an antigen. Specifically, in the present invention, the immunized patient is protected from tetanus disease or symptoms thereof.

In one embodiment, a suitable dosage is from about 1 µg to 20 µg.

Suitable routes of administration for the preparations of modified toxin described herein include, but are not limited to, direct injection. In certain embodiments, each dose is administered intramuscularly.

Dosage, toxicity, and therapeutic efficacy of the agents of the present technology can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀.

As used herein, a “therapeutically effective amount” refers to an amount of a compound that, when administered to a subject for treating a disease, is sufficient to affect such treatment for the disease. The “therapeutically effective amount” will vary depending on the compound, the disease state being treated, the severity or the disease treated, the age and relative health of the subject, the route and form of administration, the judgment of the attending medical or veterinary practitioner, and other factors. For purposes of the present invention, “treating” or “treatment” describes the management and care of a patient to combat the disease, condition, or disorder. The terms embrace both preventative, i.e., prophylactic, and palliative treatment.

The conjugated delivery platform described herein can be adjusted to target specific conditions or diseases by adjusting which conjugated modified Tet toxin platform is used.

Methods

In another aspect, provided herein are methods for engineering vaccines and conjugate vaccines having reduced toxicity and increased potency. The methods comprise genetically modifying particular amino acid residues within domains of a multi-domain protein toxin whereby the toxin is rendered incapable of expressing toxicity by either catalytic activity or receptor binding activity on target cells (e.g., neurons), is deficient in translocation, and has reduced potential for reversion to a toxic form. Through modification of multiple, independent protein functions, the methods provided herein advantageously provide full length toxins that are ideal candidates for potent vaccines and conjugate vaccines, having virtually no potential for reversion of the protein to toxicity. As used herein, the term “potency” refers to the specific ability or capacity of a vaccine, as indicated by appropriate laboratory tests or by adequately controlled clinical data, to effect protective immunity. In other words, potency is a measure of a vaccine’s strength.

In some cases, the method of obtaining an engineered bacterial protein toxoid having increased potency as a vaccine comprises or consists essentially of the following steps: selecting one or more amino acid positions in each domain of an amino acid sequence encoding a multi-domain bacterial protein toxin, where each position is selected to inactivate a protein function associated with each domain, where the domains comprise two or more of a catalytic domain, a translocation domain, a receptor binding domain, and a

substrate binding domain; substituting a native amino acid residue at each selected position with a non-native amino acid residue, whereby the substitution inactivates one or more protein functions associated with the domain; and expressing in a host cell a nucleic acid sequence encoding a full-length bacterial protein toxin comprising the substituted non-native amino acid residues, whereby the expressed protein exhibits partial or complete loss of catalytic activity, receptor binding activity, translocation activity, or substrate binding activity relative to the full-length bacterial protein toxin comprising the native amino acids.

Selecting amino acid residues for modification including analyzing protein sequence (e.g., primary amino acid sequence) or structural information to identify individual functional amino acid residues of each domain of the bacterial protein toxin. Preferably, selected residues are those one or more individual amino acid residues of each functional domain (e.g., catalytic domain, translocation domain, receptor binding domain, substrate binding domain) that can be modified without destabilizing the full-length protein and without loss of immunogenicity. Protein stability can be assessed by any appropriate method. In some cases, modified toxins are tested for stability by measuring trypsin sensitivity,³ and modified toxins are tested for immunogenicity by measuring the immune response to modified toxin vaccination in a mouse model.⁴

Protein structure information can be obtained by any appropriate method such as, for example, x-ray crystallography, electron microscopy, nuclear magnetic resonance spectroscopy, computational protein structure modeling, or a combination thereof. In some cases, the crystal structure of a bacterial protein toxin of interest can be used to identify, for example, specific sites of interaction between functional domains of the toxin and functionally conserved residues between related protein toxins. For example, using diphtheria toxin as an example, amino acid residues involved in catalysis, substrate binding, translocation, and receptor binding are identified in the literature from prior studies and engineered into the gene encoding diphtheria toxin to produce a mutated diphtheria toxin gene that encodes multiple, independent mutations in each of the four functional domains of the toxin based upon alignment of the amino acid sequence within the crystal structure of diphtheria toxin. Nucleic acid sequences encoding the mutated diphtheria toxin will be transformed into *Escherichia coli* and the protein produced recombinantly and tested for loss of toxicity, maintenance of stability, and retained immunogenicity.

By way of example, inter- and intra-domain molecular interactions can be determined from analysis of a wild-type bacterial protein toxin’s crystal structure. Substitution mutations can be achieved using well-known methods, such as site-directed mutagenesis, PCR-mediated mutagenesis, and total gene synthesis, as well as other methods known in the art. Illustrative methods of mutagenesis protocols are shown, for example, in the following Examples. In some cases, site-directed mutagenesis is used to generate mutations at single amino acid residues. In some cases, a software program such as PrimerX (available at bioinformatics.org/primerx/ on the World Wide Web) can be used to design oligonucleotide primers for site specific mutagenesis. The wild type bacterial protein toxin gene can be cloned into an expression vector to serve as a template for mutagenesis by any appropriate method such as, for example, polymerase chain reaction. Mutagenesis can be confirmed by nucleic acid sequencing. In some cases, a polynucleotide encoding a modified protein toxin is located in an expression vector. In some cases, the vector is in a host cell (e.g., a bacterial

cell, a yeast cell, eukaryotic cell). Numerous expression vectors and systems are known, both for prokaryotes and eukaryotes, and the selection of an appropriate system is a matter of choice. Expression and purification of a modified protein product of the invention can be easily performed by one skilled in the art. See, Sambrook et al., "Molecular cloning-A Laboratory Manual, second edition."

The methods are applicable to virtually any bacterial protein toxin, also known as exotoxins, which are multi-domain proteins that are secreted by bacteria and, in many cases, resemble enzymes in that they act catalytically and exhibit substrate specificity. Bacterial protein toxins include, without limitation, *botulinum* toxin, tetanus toxin, shiga toxin, diphtheria toxin, *Bordetella pertussis* toxin, *E. coli* heat-labile toxin LT, *Bacillus anthracis* toxin, *Pseudomonas* exotoxin A, anthrax toxin Lethal Factor (LF), cholera enterotoxin, and *Staphylococcus aureus* exfoliatin B. Table 4 sets forth exemplary bacterial protein toxins for which crystal structure information is available for use in designing genetically engineered recombinant, non-toxic, high potency toxoids that are particularly advantageous for vaccines and conjugate vaccines.

TABLE 4

Bacterial protein toxins that have multiple, independent functional domains and solved structure

Bacterial Toxin	AB organization	AB organization and Protein Data Bank reference of the crystal structure of the bacterial toxin (AB)PDB#
Anthrax toxin	A2-B	(A1)1K93 (A2)1J7N (B)1ACC
Cholera toxin	AB5	(AB5)1XTC
Botulinum toxin	AB	(AB)3BTA
<i>Clostridium difficile</i> toxin A	AB	(AB)4R04
<i>Clostridium difficile</i> toxin B	AB	(A)3SS1 (B)6C0B
Diphtheria toxin	AB	(AB)1SGK
Tetanus toxin	AB	(AB)5N0C
<i>E. coli</i> heat-labile enterotoxin	AB5	(AB5)1LTT
Pertussis toxin	AB5	(AB5) 1PRT
<i>P. aeruginosa</i> exotoxin A	AB	(AB)1IKQ
<i>P. aeruginosa</i> ExoS	Type III effector	(A)1HE9
<i>P. aeruginosa</i> ExoU	Type III effector	(A) 4AKX
Shiga toxin	AB5	(AB5)1DMO
Typhoid toxin	A2B5	(A2B5)4K6L
<i>Yersinia pestis</i> (plague) YopE	Type III effector	(A)1JYA

In some cases, independent, inactivating modifications are selected to disrupt one or more of a bacterial protein toxin's catalytic domain, substrate binding domain, translocation domain, and receptor-binding domain. For example, diphtheria toxin is a bacterial protein toxin comprising having a length of 535 amino acids. Inactivating amino acid residues involved with catalysis (E149S), substrate binding (H21A), translocation (E349K, E362K), and receptor binding (K516A, K526A, H391A) functions will yield a non-toxic, yet potent diphtheria vaccine.

It will be understood, however, that the methods can be applied to virtually any protein having multiple functional domains (e.g., effector domains) for which amino acid sequence and/or crystal structure information is available and for which inactivation modifications can be determined based on known structure-function properties.

In certain embodiments, genetic modifications to hinder host receptor activity are introduced into the C-terminal portion of the Heavy chain (HC_C). To hinder catalytic activity, genetic modifications are introduced into the light

chain at amino acid residues required or important for neurotransmitter release via the SNARE complex.

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

Kits.

In another aspect, provided herein is a kit for administering to a subject a vaccine or adjuvant comprising a modified toxin vaccine as described herein. In one embodiment, the kit comprises a form of a modified toxin (e.g., a modified tetanus toxin as described herein). 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 tetani*, particularly a disease caused by tetanus toxin. In one embodiment, the modified toxin of the present invention is formulated, delivered and stored for use in physiologic conditions. Suitable pharmaceutical carriers include, but are not limited to, for example, saline solution (e.g., 0.9% sodium chloride), phosphate buffer saline, lactated ringer's solution, and the like.

By "instructions for use" we mean a publication, a recording, a diagram, or any other medium of expression which is used to communicate the usefulness of the invention for one of the purposes set forth herein. The instructional material of the kit can, for example, be affixed to a container which contains the present invention or be shipped together with a container which contains the invention. Alternatively, the instructional material can be shipped separately from the container or provided on an electronically accessible form on an internet website with the intention that the instructional material and the biocompatible hydrogel be used cooperatively by the recipient.

In the specification and in the claims, the terms "including" and "comprising" are open-ended terms and should be interpreted to mean "including, but not limited to" These terms encompass the more restrictive terms "consisting essentially of" and "consisting of."

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", "characterized by" and "having" can be used interchangeably.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications and patents specifically mentioned herein are incorporated by reference in their entirety for all purposes including describing and disclosing the chemicals, instruments, statistical analyses and methodologies which are reported in the publications which might be used in connection with the invention. All references cited in this specification are to be taken as indicative of the level of skill in the art. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The invention will be more fully understood upon consideration of the following non-limiting examples.

EXAMPLES

Example 1

Isolation and Characterization of M-BoNT/A1 and M-BoNT/A1^W

This Example describes full length BoNT-engineered with defects in catalysis and receptor binding protected against challenge by 10⁶ LD₅₀ of native BoNT/A1. These data indicate the potential for genetically engineered toxins as a platform strategy with multiple mutations that reduce toxicity by independent mechanisms for development of vaccines against botulism and other toxin-mediated diseases.

Materials and Methods:

Biosafety and Biosecurity: Experiments conducted at the University of Wisconsin-Madison were approved by the Institutional Biosafety Committee. In addition, experiments were conducted in laboratories approved for this research by the Federal Select Agent Program by researchers who have undergone suitability assessments and adhere to institutional policies and practices. Animal experiments were approved and conducted according to the guidelines of the Animal Care and Use Committee at the University of Wisconsin-Madison. The Department of Health and Human Services determined that genes and protein products of BoNT/A encoding three LC mutations (E224A/R363A/Y366F), termed M) do not meet the regulatory definition of a select agent, allowing production of M-BoNT/A without select agent registration (§ 73.3 HHS select agents and toxins 42 CFR 73.3 (e)(1)).

Botulinum Neurotoxins: BoNT/A1, /A2, /A3 and /A5 were purified from *C. botulinum* strains Hall A-hyper, Kyoto-F, CDC A3 (provided by Susan Maslanka and Brian Raphael, Centers for Disease Control and Prevention) and A661222 by standard toxin purification protocols (Jacobson et al., 2011; Lin et al., 2010; Malizio et al., 2000; Tepp et al., 2012). BoNT/A6 was purified from CDC41370 B2tox⁻ (modified from strain CDC41370 to produce only BoNT/A6) toxin using previously described methods (Pellett et al., 2016). Toxin purity was confirmed by spectroscopy and SDS-PAGE analysis (Whitemarsh et al., 2013). Purified toxins were stored in phosphate buffered saline with 40% glycerol at -20° C. until use. Activities of the five subtype preparations were determined using a standard intraperitoneal mouse bioassay (MBA) as previously described (Hatheway, 1988; Schantz, 1978). The half-lethal dose of each toxin was defined as 1 mouse LD50 Unit (U). Specific activities of the BoNT/A subtypes were; 8 pg/U (A1), 7.9 pg/U (A2), 17 pg/U (A3), 7.3 pg/U (A5), and 5.9 pg/U (A6).

Recombinant BoNT Derivatives: Production of HC_C/A1 (W1266A) (HC_C/A1^W), LC/A1 (R363A/Y366F) (LC/A1^{R3}), LCHC_N/A1 (E224A/R363A/Y366F) (M-LCHC_N/A1), BoNT/A1 (E224A/R363A/Y366F) (M-BoNT/A1), BoNT/A1 (E224A/R363A/Y366F/W1266A) (M-BoNT/A1^W) and non-catalytic-Tetanus toxin (R372A/Y375F) (TeNTRY) was performed as previously described (Przedpelski et al., 2013). Briefly, *E. coli* were grown overnight on LB agar with 50 µg/ml kanamycin at 37° C. Cultures were inoculated into LB medium (400 ml) containing kanamycin for 3-6 hours with shaking at 37° C. to an OD600 of ~0.6 when 1.0 mM IPTG was added, followed by an overnight incubation with shaking at 16° C. Cells were harvested and

the pellet was suspended in lysis buffer (20 mM Tris (pH 7.9), 500 mM NaCl, 5 mM Imidazole, RNase, DNase, and protease inhibitors) (Sigma). Cells were broken with a French Press, clarified by centrifugation, and filtered through a 0.45 µm Surfactant-Free Cellulose Acetate membrane (Thermo Fischer). Lysates were further purified by tandem gravity-flow chromatography using Ni²⁺-NTA resin (Qiagen), p-aminobenzamidine-agarose (Sigma), and Strep-tactin Superflow high-capacity resin (IBA-LifeSciences). Purified proteins were dialyzed into 10 mM Tris (pH 7.9), 200 mM NaCl, and 40% glycerol and stored at -20° C. Recombinant proteins used in this study are shown (FIG. 1). The nucleotide sequence encoding M-BoNT/A1^W is set forth as SEQ ID NO:4 (see FIG. 9).

Vaccine Challenge: Groups of female ICR mice (18 to 22 g) were immunized intraperitoneally with either HC_C/A1^W, M-LCHC_N/A1, M-BoNT/A1, or M-BoNT/A1^W at the indicated concentration mixed with an equal volume of alhydrogel as an adjuvant. Non-trypsinized M-BoNT/A1 and M-BoNT/A1^W were used as vaccines. Vaccines were administered on day 1 and 14, blood was collected by maxillary bleed on day 21, and mice were challenged with BoNT/A1, BoNT/A2, or a BoNT-/A2, /A3, /A5, A6 cocktail as indicated on day 26. At least eight mice per group were used in each experiment as indicated. Results were evaluated for statistical relevance by two-tailed, paired student t-test with a p=0.05.

ELISA: BoNT derivatives or TeNT^{RXY} (250 ng/well) were added in 0.1 ml of coating buffer, 50 mM Na₂CO₃ (pH 9.6) to high protein binding 96-well plates (Corning) and incubated overnight at 4° C. Plates were then washed three times with 0.3 ml of phosphate-buffered saline (PBS) with 0.05% Tween 20 and blocked at room temperature (RT) for 30 minutes with 0.2 ml of PBS with 1% (wt/vol) bovine serum albumin (BSA). Plates were incubated at RT for 1 h with the indicated dilution of serum either 1:20,000 or 1:30,000 from individually vaccinated mice in PBS with 1% (wt/vol) BSA (0.1 ml). After washing three times with 0.3 ml PBS with 0.05% Tween 20, plates were incubated at RT for 1 hour with goat α-mouse IgG-horseradish peroxidase (IgG-HRP diluted 1:20,000; Thermo) in PBS with 1% (wt/vol) BSA. Plates were washed three times with 0.3 ml PBS with 0.05% Tween 20 and then incubated with 0.1 ml per well tetramethyl benzidine (TMB; Thermo Ultra TMB) as substrate. Reactions were terminated after 10 min with 0.1 ml of 0.1 M H₂SO₄, and absorbance was read at 450 nm. Control ELISAs, measuring bound antigens with α-HA and α-FLAG antibodies showed the presence of the appropriate epitope within each antigen, within 15% (data not shown). For the ELISA, statistical analyses were performed on groups of individually analyzed sera (N=10) based upon immunization and/or challenge conditions by two tailed, unpaired Student t test with P<0.05=*, 0.01=**, 0.001=***, and 0.0001=**** (GraphPad Prism 7). Individual sera were analyzed by at least two-independent ELISA performed in duplicate. Analysis of mouse serum at 1:20,000-1:30,000 fold dilutions was based upon assessment of several individual sera. The serum dilutions were established from a dose-response ELISA of several mice vaccinated with M-BoNT/A1 which survived challenge with BoNT/A1. A representative ELISA is shown in FIG. 6.

Cell based assay for detection of neutralizing antibodies: Cell based neutralization assays were performed as previously described (Whitemarsh et al., 2012). Briefly, human induced pluripotent stem cell (hiPSC) derived neurons (Cellular Dynamics International, WI) were seeded into poly-L-ornithine and MatrigelTM coated 96-well TPP plates (Mid-

west Scientific, MO) at a density of about 35,000-40,000 cells per well and maintained in iCell Neurons culture media (Cellular Dynamics International, WI) according to manufacturer's instructions for 7 days prior to the neutralization assay. To detect neutralizing antibodies in the mouse sera, 2 pM of BoNT/A1 was combined with serial dilutions of sterile filtered sera in culture media and incubated for 1 hour at 37° C. BoNT/A1 without sera was used as a 'no antibody' reference, and serum from naïve mice was used as a control. Serum without toxin was used as a negative control. Fifty µl of each antibody-toxin mixture was added per well of hiPSC derived neurons in at least duplicates, and cells were incubated for 24 hours at 37° C., 5% CO₂. The toxin/antibody was aspirated from the cells, and cell lysates were prepared in 50 µl of lithium dodecyl sulfate (LDS) sample buffer (Life Technologies). The cell lysates were analyzed by Western blot for SNAP-25 cleavage as previously described (Pellett et al., 2007; Pellett et al., 2010). Images were obtained using PhosphaGlo reagent (KPL, Gaithersburg, Md.) and a Fotodyne/FOTO/Analyst FX imaging system (Harland, Wis.). Cleaved (24 kDa) versus uncleaved (25 kDa) SNAP-25 signal was analyzed by densitometry using TotalLab Quant software (Fotodyne, Harland, Wis.). Percentage of protection was determined by comparison to the 'no-antibody' control, and IC₅₀ values were estimated, using GraphPad Prism 6 software and a nonlinear regression, variable slope, four parameters.

Results

M-BoNT/A1 is Not Toxic to Outbred Mice or Cells in Culture

Ten µg of either trypsinized- or non-trypsinized-M-BoNT/A1/mouse (ICR) injected intraperitoneal did not result in observable signs of botulism, indicating M-BoNT/A1 was at least one million-fold less toxic than native BoNT/A1. In addition, incubation of human iPSC derived neurons with 80 nM M-BoNT/A1 did not yield detectable SNAP-25 cleavage, while incubation with 50 fM native BoNT/A1 cleaved SNAP-25, indicating at least a million-fold lower toxicity by the cell based assay (data not shown).

M-BoNT/A1 and M-BoNT/A1^W are More Protective Vaccines than HC_C/A1^W

Vaccine challenges were conducted on outbred ICR mice (n=8-10) to reflect natural immune variance within the host (Rai et al., 2009), using a primary immunization followed by one boost. Since previous studies showed HC_C/A1 (W1266A) (HC_C/A1^W) had similar vaccine potency in the mouse model of botulism as HC_C/A1 (Przedpelski et al., 2013), M-BoNT/A1^W was also engineered. Mice vaccinated with 0.3 µg/mouse single chain M-BoNT/A1^W or 0.2 µg/mouse M-LCHC_N/A1 were protected against challenge by 10⁶ LD₅₀ of native BoNT/A1 or 10⁵ LD₅₀ of a BoNT/A subtype cocktail (2.5×10⁴ LD₅₀ each A2, A3, A5, A6) and partially protected against challenge by 10⁶ LD₅₀ of native BoNT/A2, a heterologous subtype (Table 4). Mice vaccinated with 0.1 µg/mouse HC_C/A1^W were partially protected against challenge by 10³ LD₅₀ of native BoNT/A1 or native BoNT/A2. These data indicate that at equimolar doses, the M-BoNT/A1^W and the M-LCHC_N/A1 vaccines protected against 1,000-fold more toxin than the HC_C/A1^W vaccine. In a comparison of the vaccines using weight equivalent doses, mice vaccinated with 0.3 µg/mouse of HC_C/A1^W, were partially protected against challenge by 10⁵ LD₅₀ of native BoNT/A1 or 10⁵ LD₅₀ of native BoNT/A subtype cocktail. This indicates that even at equal concentrations (and a 3-fold molar excess of HC_C), the M-BoNT/A1^W and the

M-LCHC_N/A1 vaccines protected better against homologous and heterologous BoNT/A challenge. No difference in protection of the M-BoNT/A1^W and M-BoNT/A1 vaccine were noted, indicating that the additional 'receptor binding' mutation does not affect vaccine potency. Overall, M-BoNT/A1, M-BoNT/A1^W and M-LCHC_N/A1 were more potent vaccines than HC_C/A1^W. Duration of action in cultured neurons was further investigated in human iPSC derived neurons by exposing the neurons to serial dilutions of either BoNT/A1 or BoNT/A6 for 72 hours, followed by complete removal of extracellular toxin, and further incubation in culture media. Cells with each dilution series were harvested at days 3, 39, and 70 in triplicate, and the EC₅₀ for SNAP-25 cleavage was determined for each time point. For BoNT/A6, EC₅₀ values were ~0.04, 0.7, and 1 U/50 µl/well (32, 560, and 800 fM) at days 3, 39, and 70, respectively (FIG. 2). For BoNT/A1 EC₅₀ values were ~0.7, 6.3, and 28 U/50 µl/well (313, 2940, and 12,880 fM, respectively) at days 3, 39, and 70 (FIG. 2). The half-life of BoNT/A1 and /A6 in these hiPSC derived neurons as determined from the EC₅₀ values over time was similar for both BoNT/A1 and /A6, approximately 12 days and 14 days, respectively (FIG. 2). Taken together, these results indicate that BoNT/A6 has a long duration of action, similar to other BoNT/A subtypes.

Antibody responses to BoNT vaccination varied qualitatively and quantitatively in outbred mice.

Vaccination with M-BoNT/A1^W and M-BoNT/A1 provided complete protection to challenge by 10⁵ LD₅₀ BoNT/A2 and partial protection to challenge by 10⁶ LD₅₀ of native BoNT/A2, a heterologous subtype (Table 5). Antibody responses of vaccinated mice analyzed by ELISA showed mice surviving or not surviving native BoNT/A2 challenge had similar dominant antibody titers to BoNT and LCHC_N, which were not statistically different (FIG. 2). Thus, partial protection against native BoNT/A2 challenge appears to be due to specific differences in the composition of neutralizing epitopes between BoNT/A subtypes, not the ability of the vaccinated mice to mount an immune response to the delivered vaccine.

TABLE 5

Vaccine potency of recombinant BoNT and BoNT-derivatives in the mouse model of botulism					
Vaccine Primary & Boost (µg) ^a	Challenge BoNT serotype	Survivors/Challenged Units of BoNT/A LD ₅₀ challenge (U) ^b			
		10 ³ U	10 ⁴ U	10 ⁵ U	10 ⁶ U
Experiment 1					
M-BoNT/A1 (0.3)	A1	10/10	— ^c	—	—
	A2	10/10	—	—	—
M-BoNT/A1 ^W (0.3)	A1	10/10	—	—	—
	A2	10/10	—	—	—
HC _C /A1 ^W (0.1)	A1	7/10	—	—	—
	A2	6/10	—	—	—
Alum	A1	0/5	—	—	—
Experiment 2					
M-BoNT/A1 (0.3)	A2	—	8/8	8/8	5/9
M-BoNT/A1 ^W (0.3)	A2	—	8/8	8/8	3/9
Alum	A2	—	—	—	0/5
Experiment 3					
M-BoNT/A1 ^W (0.3)	A1	—	—	—	10/10
M-LCHC _N /A1 (0.2)	A1	—	—	—	10/10
M-LCHC _N /A1 (0.2) + HC _C /A1 ^W (0.1)	A1	—	—	—	10/10

TABLE 5-continued

Vaccine potency of recombinant BoNT and BoNT-derivatives in the mouse model of botulism					
Vaccine Primary & Boost (μg) ^a	Challenge BoNT serotype	Survivors/Challenged Units of BoNT/A LD ₅₀ challenge (U) ^b			
		10 ³ U	10 ⁴ U	10 ⁵ U	10 ⁶ U
HC _C /A1 ^W (0.3)	A1	—	—	7/10	—
M-BoNT/A1 ^W (0.3)	A(subtype cocktail) ^c	—	—	10/10	—
M-LCHC _N /A1 (0.2)	A(subtype Cocktail)	—	—	10/10	—
M-LCHC _N /A1 (0.2) + HC _C /A1 ^W (0.1)	A(subtype Cocktail)	—	—	9/10	—
HC _C /A1 ^W (0.3)	A(subtype Cocktail)	—	—	7/10	—
Alum	A(subtype Cocktail)	—	0/5	—	—

^aMice were immunized IP with the indicated vaccine with a hydrogel as adjuvant. Vaccines were administered on day 1 and 14, blood was collected on day 21, and mice were challenged as indicated on day 26

^bU = One half-lethal dose of a botulinum neurotoxin at 72 h post challenge is defined as 1 mouse LD₅₀

— = not determined

^cW = W1266A mutation within the ganglioside binding domain of HC/A1

^eA(subtype cocktail) = 25,000 LD₅₀ U of BoNT/A2/A3/A5 and /A6 (total 100,000 LD₅₀ U)

Antibody responses of vaccinated mice were analyzed by ELISA of individual sera using either the LCA1^{R_Y}, HC_C/A1^W, M-LCHC_N/A1, or M-BoNT/A1 holotoxin as binding substrates. The antibody response within each group of vaccinated mice varied within the group quantitatively and qualitatively. Mice vaccinated with M-BoNT/A1^W (FIG. 3, lower left) showed dominant antibody titers to BoNT (mean titer 2.2 (range 1.3-2.6)) and LCHC_N (mean titer 1.7 (range 0.8-2.4)). Titers to HC_C varied between mice (mean titer 0.41 (range 0.07-1.83)). Titers to LC were not above controls, indicating most of the antibody response was directed towards the HC. Variance in the range of titers was due to the varied antibody titers among individual mice, not to variance in the ELISA replicates. A similar immune response to M-BoNT/A1 vaccination was observed (data not shown). Mice vaccinated with M-LCHC_N/A1 (FIG. 3, upper left) also had dominant antibody titers to BoNT and LCHC_N, with on average lower titers than mice vaccinated with M-BoNT/A1^W. Mice vaccinated with M-LCHC_N/A1+HC_C/A1^W had antibody titer profiles that were qualitatively like mice vaccinated with M-BoNT/A1^W, quantitatively they compared to mice vaccinated with M-LCHC_N/A1 alone (FIG. 7). Mice vaccinated with HC_C/A1^W had antibody titers to HC_C that correlated with survival to BoNT/A1 challenge (FIG. 3, lower right). Mice vaccinated with M-BoNT/A1^W possessed limited antibody titers to TeNT^{R_Y} (FIG. 3), indicating that the observed antibody responses were BoNT-specific.

Properties of sera from individually vaccinated mice surviving BoNT challenge. Analysis of individual sera from mice vaccinated with M-BoNT/A1^W, M-LCHC_N/A1, or HC_C/A1^W surviving native BoNT/A1 challenge showed several representative immune responses to vaccination (FIG. 4). Since our earlier studies (Przedpelski et al., 2013 *Infect Immun.* 81(7):2638-44) did not characterize the antibody response to M-LCHC_N/A1 vaccination, sera from three LCHC_N/A1 vaccinated mice were analyzed. ELISA results showed mice vaccinated with M-BoNT/A1^W had dominant antibody titers to BoNT and LCHC_N (#7) or to BoNT, LCHC_N, and HC_C (mouse #3). Mice vaccinated with M-LCHC_N/A1 showed dominant antibody responses to BoNT and LCHC_N (mice #21, #24, and #25), while mice

vaccinated with HC_C/A1^W and surviving BoNT/A1 challenge had a dominant antibody response to HC_C (mouse #78). Overall, the antibody responses to TeNT^{R_Y} were low, indicating that the immunoreactivity detected in the ELISA were specific to BoNT.

The BoNT/A and HC_C vaccines elicit stronger neutralizing antibody response than the LCHC_N vaccine.

The neutralizing antibodies in vaccinated mouse sera were measured by cell-based assay using hiPSC derived neurons. From each vaccination group of the equimolar vaccination challenge, 10 sera were pooled and tested for their ability to neutralize BoNT/A1 induced cleavage of SNAP25 in the cell based assay. M-BoNT/A1^W vaccinated pool was most potent for neutralization with an IC₅₀ value of 0.004, which was about 2-fold lower than the HC_C/A1^W vaccinated pool and the M-LCHC_N/A1+HC_C/A1^W vaccinated pool, and about 5-fold lower than the M-LCHC_N/A1 vaccinated pool (data not shown). The similarity in neutralizing antibody titers in mice vaccinated with HC_C and M-BoNT/A1 was striking considering that the M-BoNT/A1 vaccine protected mice against >1,000-fold greater toxin challenge than the HC_C vaccine. To investigate this further, the ability of six representative individual sera to neutralize BoNT/A1 cleavage of SNAP25 in a cell based assay was also determined (FIG. 5). Overall, each of the six sera neutralized BoNT/A1 action with ~10-fold difference in the serum potency. Sera from HC_C/A1^W vaccination (mouse #78) and M-BoNT/A1^W vaccination (mouse #3), which contained a dominant antibody response to HC_C (FIG. 4), were the most potent inhibitors of BoNT/A1 cleavage of SNAP-25. Sera without a detectable HC_C antibody response (mice #7, #21, #24, and #25), were less effective in inhibiting SNAP-25 cleavage. Thus, in this assay, vaccines with HC_C epitopes elicited a greater 'neutralizing/blocking antibody' response than the LCHC_N vaccine. Together, these data indicate that the HC_C domain of BoNT/A1 elicits a stronger neutralizing/blocking antibody response than the HC_N or LC domains, but that the HC_N and possibly the LC domain play a major role in in vivo protection.

Discussion

In an outbred mouse model of botulism, M-BoNT/A1, M-BoNT/A1^W and M-LCHC_N/A1 were more potent vaccines than HC_C/A1^W. Assessment of sera from vaccinated mice that survived BoNT/A1 challenge showed a common response to LCHC_N consistent with the presence of neutralizing epitopes within LCHC_N. The ability of M-BoNT/A1^W to elicit a similar protective immune response relative to M-BoNT/A1 showed reduction of host cell binding did not negatively affect vaccine efficacy. Thus, full-length BoNT engineered with defects in both the catalytic and receptor binding domains represents a novel platform strategy for development of vaccines against botulism and other toxin-mediated diseases. Collier and coworkers (Killeen et al., 1992) showed the ability to generate second-site mutations that partially reverted a genetically inactivated diphtheria toxin as a test for vaccine development. In addition, recent studies by Smith and coworkers show a need for greater attenuation than only reduction of catalysis for several serotypes of BoNT-based vaccines (Webb et al., 2017).

In an earlier study, LCHC_N was described as a BoNT vaccine candidate (Shone et al., 2009). LCHC_N was produced in high amounts by *E. coli* by fermentation and was effective as a single dose vaccine to a low dose BoNT challenge (10³ LD₅₀ of BoNT). In this report, we observed that LCHC_N was a potent vaccine by direct comparison to other BoNT vaccine candidates, using a primary immunization with one boost and confirmed the presence of neu-

tralizing epitope(s) within LCHCN (Shone et al., 2009). Dolly and colleagues identified a LC specific monoclonal antibody (Mab) that prevented BoNT/A action (Cenci Di Bello et al., 1994), while Marks and colleagues identified BoNT/A neutralizing mAbs with LC function that inhibited SNARE cleavage (Cheng et al., 2009) and mAbs that targeted HC_N and neutralized several BoNT serotypes (Garcia-Rodriguez et al., 2011). Together, these studies indicate that BoNT vaccination elicits the production of antibodies to neutralizing epitopes within the LCHC_N domains. Since M-BoNT/A1^W elicited a greater antibody response than M-LCHC_N/A1 (FIG. 7), along with the determination that HC_C produced antibodies with the greatest neutralizing/blocking potencies in cultured cells, vaccines that include HC_C, such as M-BoNT/A1^W, would be expected to be more protective in a 'high-dose' exposure scenario than LCHC_N or HC_C vaccine derivatives.

The HC_C is a popular domain to develop vaccines against botulism, using DNA- and viral-vectors, as well as protein-base vaccines built upon earlier studies showing neutralizing potency of the HC_C (Clayton et al., 1995) and ease of production (Baldwin et al., 2008). Smith and colleagues expressed HC_C in the yeast, *Pichia pastoris*, and reported protective immunity elicited by HC(c) (Byrne and Smith, 2000) and subsequently, a bivalent vaccine composed of recombinant HC_C/A and HC_C/B (rBV A/B), which is now in clinical trial (Webb and Smith, 2013). *E. coli* has also been used as a heterologous host for BoNT vaccine development, including production of a seven serotype (A-G) HC_C-vaccine against BoNT challenge (Baldwin et al., 2008). To enhance vaccine potency a mutation was introduced to the HC_C blocking host receptor binding, where HC_C^W retained vaccine potency (Przedpelski et al., 2013). While ease of production makes HC_C an attractive vaccine platform, the current study showed M-BoNT/A1^W was a more potent vaccine than HC_C/A1^W. This is supported by the finding of Atassi and colleagues, who detected immune epitopes within the LC and HC_N, using human serum from cervical dystonia patients resistant to BoNT therapy (Atassi et al., 2011; Dolimbek et al., 2007), consistent with the immunogenicity of the LCHC_N of BoNT.

A recent study by Smith and coworkers (Webb et al., 2017) reported that catalytically inactive BoNT showed greater potency to challenge by 1000 LD₅₀ toxin challenge after single vaccination than the corresponding HC_C. The challenge experiments described by Smith coworkers measured threshold toxin challenges, which differed from the current study that measures protection to endpoint toxin challenge. The data showed that in both cases, either measuring protection to toxin challenge by threshold or endpoint, full-length BoNT vaccines were more potent than their respective HC_C subunits. M-BoNT/A1^W, with defects in catalysis and host receptor binding, was effective in the endpoint toxin challenges relative to the subunit. By inactivation of multiple functional sites to lower the potential toxicity due to cell binding or entry, utility of M-BoNT/A1^W as a vaccine candidate addresses a concern that genetic inactivation of catalytic function alone may not provide a sufficient margin of safety for vaccine development of full-length BoNTs (Webb et al., 2017).

While the utility of the HC_C as a vaccine candidate against botulism is established (Baldwin et al., 2008; Henderson, 2006), the current study shows multi-domain derivatives of BoNT are more potent vaccines than HC_C. M-BoNT/A1^W elicited a common dominant antibody response to LCHC_N, but a varied HC_C antibody response in outbred mice. The ability to reduce both catalysis and receptor binding support the use of M-BoNT/A1^W as a vaccine platform against botulism. Protection against a BoNT/A subtype cocktail confirmed broad neutralization capacity of this vaccine. M-BoNT/A1^W used as a vaccine in this study was not processed to an activated, di-chain form and toxicity was not detected in mice or cells, suggesting single chain M-BoNT/A1^W as a safe and effective vaccine.

Example 2

Characterization of Disrupted Light Chain Translocation in Lysine768 TeNT Variants

This section describes the identification and characterization for the first time of a single amino acid point mutation within the translocation domain of TT that blocked light chain (LC) translocation. Identification of a role for K768 in LC translocation provides, for the first time, an opportunity to inactivate independent activities of TT, and by analogy BT (see Table 6), catalysis, translocation, and receptor binding, for recombinant vaccine development.

Using tetanus toxin, we recently identified a rate limiting step in Light Chain (LC) translocation encoded within the translocation domain. Lysine (K) 768 is located in a loop that links two long α -helices (helix12-13 and helix16-17). Site directed mutagenesis identified a point mutation K768A, located within the loop that connects the two long α -helices of the translocation domain that inhibited translocation (FIG. 12). Cell studies showed that M-TT(K768A) was did not bind neuronal membranes, which supports a role for the loop in membrane penetration. Control experiments showed that the K768A mutation did not inhibit TT binding, entry, trafficking, or pore formation in hosts cell and did not inhibit the preferred cleavage of Light Chain (LC)-Heavy Chain (HC) by trypsin, indicating that the mutation did not disturb overall M-TT structure and implicating a direct role for the loop in light chain translocation. Other experiments indicated that K768 was not a component of the pH trigger. This is the first single amino acid within the translocation domain that is required to Light Chain translocation and implicates a role for the two long α -helices (helix12-13 and helix16-17) in toxin-membrane interactions. Other experiments demonstrated that a D767A/E769A mutation also yielded a translocation defect in tetanus toxin, making D767/E769 complementary and/or additive to K768 for inhibiting LC translocation in tetanus toxin.

Engineering independent mutations into *botulinum* toxin (BT) (see Table 6) and TT vaccine candidates will inactivate each of the three functions of toxin action: catalysis, translocation, and receptor binding, thus enhancing vaccine safety. Multiple, independent mutations reduce toxin potency exponentially, enhancing vaccine safety without disturbing protein structure and potentially immunogenicity, and reduce reversion potential during large scale production.

TABLE 6

Exemplary point mutations for 8M-BT/A					
M-BT/A	Zn ⁺⁺ binding	Substrate Binding and catalysis	Light Chain translocation	Protein receptor binding	Ganglioside receptor binding
	E224Q, R363A, Y366F Termed 3M-BT/A and is >10 ⁵ -fold less toxic than BT WT	L174A, E370A This double mutation is 4X10 ⁴ -fold less catalytic than LC/AWT	K758A Inhibits LC translocation (preliminary data)	R1156A This mutation Inhibits 5V2 binding,	W1266A This mutation is ~800-fold less toxic than BT WT
4M-BT/A	E224Q, R363A, Y366F				W1266A
5M-BT/A	E224Q, R363A, Y366F			R1156A	W1266A
6M-BT/A	E224Q, R363A, Y366F		K758A	R1156A	W1266A
7M-BT/A	E224Q, R363A, Y366F	D370A	K758A	R1156A	W1266A
8M-BT/A	E224Q, R363A, Y366F	L174A, D370A	K758A	R1156A	W1266A

Example 3

Engineered M-Tetanus Toxin (M-TT) as a Low Dose Protective Vaccine

Organized like BT, Tetanus toxin (TT) is an AB toxin that comprises an N-terminal domain (catalytic Light Chain, LC)

translocation (K768A) (FIG. 14). 6M-TT contains mutations in each of the tetanus toxin functions: catalysis, translocation, and receptor binding. 6M-TT was purified from *E. coli* at 6 mg/liter of batch culture. Four mice each injected with 20 µg of single chain or di-chain 6M-TT did not show any symptoms of tetanus. Thus, 6M-TT is a soluble, well-expressed, and non-toxic protein.

TABLE 7

Exemplary point mutations for 8M-TT				
	Zn ⁺⁺ binding	Substrate Binding & catalysis	Light Chain translocation	Ganglioside receptor binding
	E234Q, R372A, Y375F (TT(R372A, Y375F, 2M-TT) is 125,000-fold less toxic than native TT ⁴²)	Y26A, L231K	K768A Inhibits LC translocation (preliminary data)	R1226L, W1289A This mutation is ~800-fold less toxic than TT WT
2MTT	R372A, Y375F			
5M-TT	E234Q, R372A, Y375F			R1226L, W1289A
6M-TT	E234Q, R372A, Y375F		K768A (or D767A or E769A)	R1226L, W1289A
7M-TT	E234Q, R372A, Y375F	L231K	K768A (or D767A or E769A)	R1226L, W1289A
8M-TT	E234Q, R372A, Y375F	Y26A, L231K	K768A (or D767A or E769A)	R1226L, W1289A

and a C-terminal domain (translocation and receptor binding Heavy Chain, HC). Mutation of two amino acids within the Zn⁺⁺ binding pocket of TT(R372A, Y375F) yielded 2M-TT that inhibited Zn⁺⁺ binding and reduced toxicity by 125,000-fold relative to native tetanus toxin. The 2M-TT amino acid sequence is set forth as SEQ ID NO:2, with the nucleotide sequence set forth as SEQ ID NO:3. In preliminary experiments to further reduce toxicity, an additional mutation to extend the inhibition of Zn⁺⁺ binding (E234Q) was added based upon E234 stabilizing H233, which directly coordinates Zn⁺⁺ binding. Next, neuron binding to dual ganglioside receptor binding was inhibited by engineering two independent mutations (R1226L, W1289A), producing 5M-TT (see Table 7). As a proof of principle, we engineered 6M-TT with an additional mutation to inhibit LC

6M-TT will be further engineered to, sequentially, inhibit binding and cleavage of VAMP-2 by introducing mutations at positions Y26 and L231 mutations, producing 7M-TT and 8M-TT L231 was chosen based upon earlier studies that showed this mutation reduced the kcat without affecting VAMP-2 affinity and since the L231K mutation did not affect the overall structure of the LC⁹. Y26 was chosen based upon location within the S7 pocket of HCR/T and evidence that the Y26A mutation reduced the LC/T affinity for VAMP-2⁹ (see Table 7). 8M-TT and the intermediate products (6M-TT, and 7M-TT) will be subjected to circular dichroism to measure secondary structure, trypsin sensitivity to measure overall protein stability, and mass spectrometry to measure protein composition⁴²

Since we now know that 6M-TT is produced as a soluble protein, is highly expressed, and a 20 µg injection is non-

toxic in mice, toxicity of 6M-TT, 7M-TT, and 8M-TT will first be assessed in human neuronal cell-based assays, analyzing VAMP-2 cleavage after cell entry as well as VAMP-2 cleavage in cell lysates⁶⁴. If no cleavage or cytotoxicity is detected, absence of in vivo toxicity will be established in the mouse model, using outbred female ICR mice (18 to 22 g, 5 mice/group) (Table 8).

TABLE 8

Residual Toxicity of 8M-TT	
IP injection of 8M-TT (μg)	+3 \rightarrow +14 day-post Injection
0	Score for survival
20	Observe pathology
50	Weight loss
250	Signs of stress
1000	Major organ damage Tetanus symptoms

Initial experiments will inject mice intraperitoneally with 20, 50, 250, or 1000 μg of 8M-TT per mouse (by weight 1000 μg equals $\sim 4 \times 10^7$ LD₅₀ of wild-type tetanus toxin)⁶⁵. Injected mice will be scored for survival for 3 days in the mouse bioassay and observed for up to 14 days for any symptoms indicating TT pathology including, no weight gain, signs of stress, organ damage, and tetanus symptoms. Male mice will be tested for gender disparities.

Outbred female ICR mice (8/ group) will be immunized with 0.01-0.1 μg of optimized M-TT or equivalent amounts of chemically inactivated tetanus toxoid, followed on day 14 with a boost vaccination (Table 9). On day 26, mice are bled and on day 30 mice are challenged with 10^3 - 10^6 U of tetanus toxin. To investigate long term protection, vaccinated mice will be maintained for 180 days and challenged with TT to test for the duration of immune response. Mice surviving 3 days are scored protected. Serum obtained prior to challenge will be tested for anti-TT by ELISA as previously described⁶ and for neutralizing potency against tetanus toxin intoxication of cultured neurons, as an inhibition of VAMP-2 cleavage⁷¹⁻⁷². Male mice will be tested to confirm no gender disparities.

TABLE 9

Vaccine (μg)	Days post primary immunization		
	14	26 & 176	30 & 180
Allhydrogel alone	Boost	Collect	Challenge
8M-TT (0.01-0.1 μg)	Vaccination	blood	with 10^3 - 10^6
Chemically inactivated TT (0.01-0.1 μg)			U TT

⁴Mice are vaccinated IP with two doses of M-TT primary (day 0) and boost (day 14). At day 22 and 176, mice are bled (Ig titers to TT), challenged with 10^3 - 10^6 U TT on day 26 and 180, and scored for survival for 3 days.

We expect that each independent LC point mutation introduced will have a multiplicative decrease on the catalytic activity and have reflective reductions in M-TT toxic potency, since the LC mutations inhibit independent steps in catalysis. We do not anticipate that these LC or HC mutations will affect protein stability or immunogenicity, based upon earlier studies on their effect as individual mutations in LC-TT or HC-TT. The injection of 1000 μg of 8M-TT is unlikely to be toxic in our mouse model and 8M-TT will likely have a more neutralizing immune response relative to chemically-inactivated TT, allowing low dose immunization with 8M-TT.

A recent review estimated only a fraction of the potential immunization potency of current conjugate vaccines has been achieved⁴. Studies of microbial pathogens continue to identify additional immunogens that require conjugation to protein toxoids to produce effective T-cell dependent immune responses. These include, but are not limited to, capsules of the meningococcus,¹⁵ fungi,¹⁶ and the pneumococcus¹⁷⁻¹⁸. In addition, synthetic glycans provide a promising future alternative to natural polysaccharides-based vaccines, which vary in purity and content²⁰⁻²². Tetanus toxoid is an immunogenic carrier protein²³, for polysaccharides. Tetanus toxin is among the best candidates to develop these next generation recombinant conjugate vaccines based upon our knowledge of TT structure-function properties and baseline information of tetanus toxin as a chemically inactivated toxoid, and the continued global need for tetanus vaccination. The production of a safe, easy-to-produce, and protective recombinant TT vaccine, for the first time, enables analysis of recombinant, full length atoxic (non-toxic) TT as a conjugate vaccine carrier. There currently is a lack of knowledge on the protective properties of conjugate vaccine carriers, including tetanus toxoid⁷³. The recombinant atoxic M-TT can be used as a carrier of several commonly used antigens to measure the enhanced immune response to the antigens when conjugated to M-TT versus chemically inactivated TT.

The protocol for conjugating oligosaccharides to M-TT follow published protocols of the Lees laboratory⁷⁴. Briefly, polysaccharides (PS) are reduced to a molecular weight of 100-300 kDa using an LV-1 microfluidizer. PS are prepared at 5 mg/ml in water and activated with 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP, 0.5 mg/mg)⁷⁴. An equal weight of protein (5 mg/ml) is added and the solution maintained at pH 9. The reaction is monitored by size exclusion chromatography (SEC) HPLC and quenched with excess glycine. Conjugates are purified by SEC and molecular weight determined by SEC-Multi-Angle Light Scattering. Authenticated polysaccharides and peptides will be crosslinked to 8M-TT: (i) Group B streptococcus (GBS) polysaccharide serotypes Ia, Ib, II, III, IV and IV; (2) Poly- β -(1-6)-N-acetyl-glucosamine (PNAG) which mediates biofilm formation as a candidate broad spectrum vaccine for *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, and the *Burkholderia cepacia* complex (BCC)⁴⁵; (3) Peptides currently being tested in flu vaccines.

Outbred female ICR mice (8/ group) will be immunized with 0.01-0.1 μg of conjugated-optimized M-TT or equivalent amounts of chemically inactivated tetanus toxoid, followed on day 14 with a boost vaccination (Table 10). On day 26 mice are bled, and on day 30 mice are challenged with 10^3 - 10^6 U of tetanus toxin. To investigate long term protection, vaccinated mice will be maintained for 180 days and challenged with TT to test for the duration of immune response. Mice surviving 3 days are scored protected. Serum obtained prior to challenge will be tested for anti-conjugate and anti-TT by ELISA as previously described⁶⁷⁴. Male mice will be tested for gender disparities.

TABLE 10

Vaccine (μg)	Day post primary immunization		
	14	26 or 176	30 or 180
Conjugated-8M-TT (0.01 or 0.1 μg)	Boost	Collect blood	Challenge
Conjugated-chemically inactivated TT (0.01 or 0.1 μg)	Vaccination	Assess α -conjugate and α -8M-	with 10^3 - 10^6 U TT

TABLE 10-continued

Potency of a protective conjugate 8M-TT vaccine ⁴			
Vaccine (μ g)	Day post primary immunization		
	14	26 or 176	30 or 180
Equivalent conjugate antigen, alone		TT IgM and IgG titers	
Alum (control)			

⁴ Mice are vaccinated IP with conjugate-M-TT (primary and boost). At day 21, mice are bled (Ig titers) and challenged with 10^5 or 10^6 U TT on day 30. Mice are challenged with 10^5 or 10^6 U BT/A and scored for survival for 3 days. In a separate experiment, vaccinated mice are held and on day 176 bled to measure Ig titers, T Helper Cells and B Memory and then challenged with native TT.

The PNAG oligosaccharide, GBS polysaccharide, and flu peptides will be individually conjugated to 8M-TT vaccine. Subsequent experiments will combine GBS and PNAG to produce a multiplex polysaccharide conjugate-8M-TT vaccine. Next, the flu peptides and PNAG will be combined to make a multiplex-peptide-polysaccharide conjugate-8M-TT vaccine. These experiments will test the potential of 8M-TT as a vaccine carrier.

It is expected that individual conjugated vaccines, PNAG-8M-TT, GBS-8M-TT, and peptide-8M-TT, will elicit similar immune response to the conjugate and stronger immune response to TT relative to the respective conjugate-chemically inactivated TT vaccine. We also anticipate that the immune response to polysaccharides and peptide within the 8M-TT vaccine will elicit a similar immune response relative to when the individual antigens are conjugated to 8M-TT. We anticipate the immune response to 8M-TT will correlate with protection to native TT challenge.

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- It should be noted that the above description, attached figures and their descriptions are intended to be illustrative and not limiting of this invention. Many themes and variations of this invention will be suggested to one skilled in this art, in light of the disclosure. All such themes and variations are within the contemplation hereof. For instance, while this invention has been described in conjunction with the various exemplary embodiments outlined above, various alternatives, modifications, variations, improvements, and/or substantial equivalents, whether known or that rare or may be presently unforeseen, may become apparent to those having at least ordinary skill in the art. Various changes may be made without departing from the spirit and scope of the invention. Therefore, the invention is intended to embrace all known or later-developed alternatives, modifications, variations, improvements, and/or substantial equivalents of these exemplary embodiments.

SEQUENCE LISTING

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Gly	Ser	Ala	Glu	Ile	Thr	Gly	Leu	Gly	Ala	Ile	Arg	Glu	Asp	Asn	
	1055					1060					1065				
Asn	Ile	Thr	Leu	Lys	Leu	Asp	Arg	Cys	Asn	Asn	Asn	Asn	Gln	Tyr	
	1070					1075					1080				
Val	Ser	Ile	Asp	Lys	Phe	Arg	Ile	Phe	Cys	Lys	Ala	Leu	Asn	Pro	
	1085					1090					1095				
Lys	Glu	Ile	Glu	Lys	Leu	Tyr	Thr	Ser	Tyr	Leu	Ser	Ile	Thr	Phe	
	1100					1105					1110				
Leu	Arg	Asp	Phe	Trp	Gly	Asn	Pro	Leu	Arg	Tyr	Asp	Thr	Glu	Tyr	
	1115					1120					1125				
Tyr	Leu	Ile	Pro	Val	Ala	Ser	Ser	Ser	Lys	Asp	Val	Gln	Leu	Lys	
	1130					1135					1140				
Asn	Ile	Thr	Asp	Tyr	Met	Tyr	Leu	Thr	Asn	Ala	Pro	Ser	Tyr	Thr	
	1145					1150					1155				
Asn	Gly	Lys	Leu	Asn	Ile	Tyr	Tyr	Arg	Arg	Leu	Tyr	Asn	Gly	Leu	
	1160					1165					1170				
Lys	Phe	Ile	Ile	Lys	Arg	Tyr	Thr	Pro	Asn	Asn	Glu	Ile	Asp	Ser	
	1175					1180					1185				
Phe	Val	Lys	Ser	Gly	Asp	Phe	Ile	Lys	Leu	Tyr	Val	Ser	Tyr	Asn	
	1190					1195					1200				
Asn	Asn	Glu	His	Ile	Val	Gly	Tyr	Pro	Lys	Asp	Gly	Asn	Ala	Phe	
	1205					1210					1215				

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Asn Asn Leu Asp Arg Ile Leu Arg Val Gly Tyr Asn Ala Pro Gly
 1220 1225 1230
 Ile Pro Leu Tyr Lys Lys Met Glu Ala Val Lys Leu Arg Asp Leu
 1235 1240 1245
 Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp Lys Asn Ala
 1250 1255 1260
 Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp
 1265 1270 1275
 Pro Asn Arg Asp Ile Leu Ile Ala Ser Asn Trp Tyr Phe Asn His
 1280 1285 1290
 Leu Lys Asp Lys Ile Leu Gly Cys Asp Trp Tyr Phe Val Pro Thr
 1295 1300 1305
 Asp Glu Gly Trp Thr Asn Asp
 1310 1315

<210> SEQ ID NO 3
 <211> LENGTH: 3945
 <212> TYPE: DNA
 <213> ORGANISM: Clostridium tetani

<400> SEQUENCE: 3

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 atgatggaac cgccgtattg caaaggcctg gatatttatt ataaagcgtt taaagttacc 120
 gatcgtatth ggattgtgcc ggaacgttat gaatttggca ccaaaccgga agatttcaac 180
 ccgccgagca gcctgattga aggcgcgagc gaattattatg atccgaacta tctgcgtacc 240
 gatagcgata aagatcgtht cctgcagacc atggtgaaac tgtttaaccg tattaagaac 300
 aacgtggcgg gcgaagcgtc gctggataaa attattaacg cgattccgta tctgggcaac 360
 agctatagcc tgctggataa atttgatacc aacagcaaca gcgtgagcct taacctgctg 420
 gaacaagatc cgagcggcgc gaccaccaa agcgcgatgc tgaccaacct gattattttc 480
 ggcccggggc cggtgctgaa caaaaacgaa gtgcgtggca ttgtgctgcg tgtggataac 540
 aagaactatt tcccgtagcc tgatggcttt ggcagcatta tgcagatggc gttttgcccg 600
 gaatatgtgc cgaccttga taacgtgatt gaaaacatta ccagcctgac cattggcaaa 660
 agcaaatatt tccaagatcc ggcgctgctg ctgatgcatg aactgattca tgtgctgcat 720
 ggcctgtatg gcatgcaggt gagcagccat gaaattattc cgagcaaca ggaatttat 780
 atgcagcata cctatccgat tagcgcggaa gaactgttta ctttggcgg ccaggatgcy 840
 aacctgatta gcattgatat taagaacgat ctgtatgaaa agaccctgaa cgattataaa 900
 gcgattgcga acaaaactgag ccaggtgacc agctgcaacg atccgaacat tgatattgat 960
 agctataaac agatttatca gcagaaatat cagtttgata aagatagcaa cgccagtat 1020
 attgtgaacg aagataaatt tcagattctg tataacagca ttatgtatgg ctttaccgaa 1080
 attgaactgg gcaagaatt taacattaaa accgcgctga gcttttttag catgaacct 1140
 gatccggtga aaattccgaa cctgctggat gataccattt ataacgatac cgaaggcttt 1200
 aacattgaaa gcaaaagcct gaaaagcgaa tataaaggcc agaacatgcy tgtgaacacc 1260
 aacgcgtttc gtaactgga tgatccggc ctggtgagca aactgattgg cctgtgcaag 1320
 aagattattc cgccgaccaa cattcgtgag aacctgtata accgtaccgc gagcctgacc 1380
 gatctgggcy gcgaactgtg cattaagatt aagaacgaag atctgacctt tattgcygag 1440
 aagaacagct tttagcgaaga accgtttcag gatgaaattg tgagctataa caccaagaac 1500

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aaaccgctga	actttaacta	tagcctggat	aaaattattg	tggattataa	cctgcagagc	1560
aagattaccc	tgccgaacga	tcgtaccacc	ccggtgacca	aaggcattcc	gtatgcgcgcg	1620
gaatataaga	gcaacgcggc	gagcaccatt	gaaattcata	acattgatga	taacaccatt	1680
tatcagtatc	tgtatgcgca	gaagagcccg	accaccctgc	agcgtattac	catgaccaac	1740
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agcaaagtga	accaggggcg	gcagggcatt	ctgtttctgc	agtgggtgcg	tgatattatt	1860
gatgatttta	ccaacgaaa	cagccagaaa	accaccattg	ataaaattag	cgatgtgagc	1920
accattgtgc	cgatatattg	cccggcgcgtg	aacattgtga	aacagggcta	tgaaggcaac	1980
tttattggcg	cgctggaaac	cacggcgcgtg	gtgctgctgc	tggaaatata	tccggaaatt	2040
accctgccgg	tgattgcggc	gctgagcatt	gcgaaaagca	gcacccagaa	agagaagatt	2100
attaanaacca	ttgataactt	tctggagaaa	cgttatgaga	aatggattga	agtgataaaa	2160
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atgtatcgta	gcctggaata	tcaggtggat	gcgattaaga	aaattattga	ttatgaatat	2280
aagatttata	gcgcccggga	taaagaacag	attgcggatg	aaattaacaa	cctgaaaaac	2340
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gatacccaga	gcaagaacat	tctgatgcag	tataatgaa	gcaacagcaa	atttatggc	2520
attaccgaac	tgaagaact	ggaaagcaaa	attaacaaag	tgtttagcac	cccgattccg	2580
tttagctata	gcaagaacct	ggattgctgg	gtggataacg	aagaagatat	tgatgtgatt	2640
ctgaagaaga	gcaccattct	gaacctggat	attaacaacg	atattattag	cgatattagc	2700
ggcttcaaca	gcagcgtgat	tacotatccg	gatgcgcagc	tggtaccggg	cattaacggc	2760
aaagcgattc	atctggtgaa	caacgaaagc	agcgaagtga	ttgtgcataa	agcgtggat	2820
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atttggaacc	tgaagatag	cgcgggcgaa	gtgcgtcaga	ttacctttcg	tgatctgccg	3060
gataagttta	acgcgatctc	ggcgaacaaa	tgggtgttta	ttaccattac	caacgatcgt	3120
ctgagcagcg	cgaacctgta	tattaacggc	gtgctgatgg	gcagcgcgga	aattaccggc	3180
ctgggcgcga	ttcgtgaaga	taacaacatt	accctgaaac	tggatcgttg	caacaataac	3240
aaccagtatg	tgagcattga	taaatttcgt	atTTTTTgca	aagcgtgaa	cccgaagaa	3300
attgaaaaac	tgtataccag	ctatctgagc	attacctttc	tgctgtgatt	ttggggcaac	3360
ccgctgcggt	atgataccga	atattatctg	attccggtgg	cgagcagtag	caaagatgtg	3420
cagctgaaga	acattaccga	ttatatgtat	ctgaccaacg	cgccgagcta	taccaacggc	3480
aaactgaaca	ttactatcg	tcgtctgtat	aacggcctga	aattcattat	taaacgttat	3540
accccgaaata	acgaaattga	tagctttgtg	aaaagcggcg	atTTTattaa	actgtatgtg	3600
agctataaca	ataacgaaca	tattgtgggc	tatccgaaag	atggcaacgc	gtttaataac	3660
ctggatcgta	ttctgcgtgt	gggctataac	gcgcccggca	ttccgctgta	taagaagatg	3720
gaagcgggtga	aactgogtga	tctgaaaacc	tatagcgtgc	agctgaaact	gtatgatgat	3780
aagaacgcga	gcctgggcct	ggttggaaac	cataacggtc	agattggcaa	cgatccaaac	3840

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 cgtgatattc tggattgag caactgggat ttttaaccatc tgaagacaa gatcctgggc 3900

tgtgattggt acttcgttcc gacagatgaa ggctggacca acgat 3945

<210> SEQ ID NO 4

<211> LENGTH: 1315

<212> TYPE: PRT

<213> ORGANISM: Clostridium tetani

<400> SEQUENCE: 4

Met Pro Ile Thr Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn Asn
 1 5 10 15
 Asp Thr Ile Ile Met Met Glu Pro Pro Tyr Cys Lys Gly Leu Asp Ile
 20 25 30
 Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Val Pro Glu
 35 40 45
 Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn Pro Pro Ser Ser
 50 55 60
 Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro Asn Tyr Leu Arg Thr
 65 70 75 80
 Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr Met Val Lys Leu Phe Asn
 85 90 95
 Arg Ile Lys Asn Asn Val Ala Gly Glu Ala Leu Leu Asp Lys Ile Ile
 100 105 110
 Asn Ala Ile Pro Tyr Leu Gly Asn Ser Tyr Ser Leu Leu Asp Lys Phe
 115 120 125
 Asp Thr Asn Ser Asn Ser Val Ser Phe Asn Leu Leu Glu Gln Asp Pro
 130 135 140
 Ser Gly Ala Thr Thr Lys Ser Ala Met Leu Thr Asn Leu Ile Ile Phe
 145 150 155 160
 Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu
 165 170 175
 Arg Val Asp Asn Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser
 180 185 190
 Ile Met Gln Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn
 195 200 205
 Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe
 210 215 220
 Gln Asp Pro Ala Leu Leu Leu Met His Gln Leu Ile His Val Leu His
 225 230 235 240
 Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys
 245 250 255
 Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu
 260 265 270
 Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile Asp Ile Lys
 275 280 285
 Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys Ala Ile Ala Asn
 290 295 300
 Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp
 305 310 315 320
 Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser
 325 330 335
 Asn Gly Gln Tyr Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn
 340 345 350
 Ser Ile Met Tyr Gly Phe Thr Glu Ile Glu Leu Gly Lys Lys Phe Asn

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355					360					365					
Ile	Lys	Thr	Ala	Leu	Ser	Phe	Phe	Ser	Met	Asn	His	Asp	Pro	Val	Lys
370						375					380				
Ile	Pro	Asn	Leu	Leu	Asp	Asp	Thr	Ile	Tyr	Asn	Asp	Thr	Glu	Gly	Phe
385					390					395					400
Asn	Ile	Glu	Ser	Lys	Asp	Leu	Lys	Ser	Glu	Tyr	Lys	Gly	Gln	Asn	Met
				405					410					415	
Arg	Val	Asn	Thr	Asn	Ala	Phe	Arg	Asn	Val	Asp	Gly	Ser	Gly	Leu	Val
			420					425						430	
Ser	Lys	Leu	Ile	Gly	Leu	Cys	Lys	Lys	Ile	Ile	Pro	Pro	Thr	Asn	Ile
		435				440							445		
Arg	Glu	Asn	Leu	Tyr	Asn	Arg	Thr	Ala	Ser	Leu	Thr	Asp	Leu	Gly	Gly
	450					455						460			
Glu	Leu	Cys	Ile	Lys	Ile	Lys	Asn	Glu	Asp	Leu	Thr	Phe	Ile	Ala	Glu
465				470					475						480
Lys	Asn	Ser	Phe	Ser	Glu	Glu	Pro	Phe	Gln	Asp	Glu	Ile	Val	Ser	Tyr
				485					490						495
Asn	Thr	Lys	Asn	Lys	Pro	Leu	Asn	Phe	Asn	Tyr	Ser	Leu	Asp	Lys	Ile
			500					505					510		
Ile	Val	Asp	Tyr	Asn	Leu	Gln	Ser	Lys	Ile	Thr	Leu	Pro	Asn	Asp	Arg
		515						520					525		
Thr	Thr	Pro	Val	Thr	Lys	Gly	Ile	Pro	Tyr	Ala	Pro	Glu	Tyr	Lys	Ser
		530				535						540			
Asn	Ala	Ala	Ser	Thr	Ile	Glu	Ile	His	Asn	Ile	Asp	Asp	Asn	Thr	Ile
545						550					555				560
Tyr	Gln	Tyr	Leu	Tyr	Ala	Gln	Lys	Ser	Pro	Thr	Thr	Leu	Gln	Arg	Ile
			565						570					575	
Thr	Met	Thr	Asn	Ser	Val	Asp	Asp	Ala	Leu	Ile	Asn	Ser	Thr	Lys	Ile
			580					585					590		
Tyr	Ser	Tyr	Phe	Pro	Ser	Val	Ile	Ser	Lys	Val	Asn	Gln	Gly	Ala	Gln
		595					600					605			
Gly	Ile	Leu	Phe	Leu	Gln	Trp	Val	Arg	Asp	Ile	Ile	Asp	Asp	Phe	Thr
	610					615						620			
Asn	Glu	Ser	Ser	Gln	Lys	Thr	Thr	Ile	Asp	Lys	Ile	Ser	Asp	Val	Ser
625				630							635				640
Thr	Ile	Val	Pro	Tyr	Ile	Gly	Pro	Ala	Leu	Asn	Ile	Val	Lys	Gln	Gly
				645					650					655	
Tyr	Glu	Gly	Asn	Phe	Ile	Gly	Ala	Leu	Glu	Thr	Thr	Gly	Val	Val	Leu
			660					665					670		
Leu	Leu	Glu	Tyr	Ile	Pro	Glu	Ile	Thr	Leu	Pro	Val	Ile	Ala	Ala	Leu
		675						680					685		
Ser	Ile	Ala	Glu	Ser	Ser	Thr	Gln	Lys	Glu	Lys	Ile	Ile	Lys	Thr	Ile
	690					695						700			
Asp	Asn	Phe	Leu	Glu	Lys	Arg	Tyr	Glu	Lys	Trp	Ile	Glu	Val	Tyr	Lys
705						710					715				720
Leu	Val	Lys	Ala	Lys	Trp	Leu	Gly	Thr	Val	Asn	Thr	Gln	Phe	Gln	Lys
				725					730					735	
Arg	Ser	Tyr	Gln	Met	Tyr	Arg	Ser	Leu	Glu	Tyr	Gln	Val	Asp	Ala	Ile
			740					745					750		
Lys	Lys	Ile	Ile	Asp	Tyr	Glu	Tyr	Lys	Ile	Tyr	Ser	Gly	Pro	Asp	Lys
		755						760				765			
Glu	Gln	Ile	Ala	Asp	Glu	Ile	Asn	Asn	Leu	Lys	Asn	Lys	Leu	Glu	Glu
	770							775						780	

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Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser
 785 790 795 800
 Ser Arg Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln
 805 810 815
 Leu Leu Glu Phe Asp Thr Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile
 820 825 830
 Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Lys Lys Leu Glu
 835 840 845
 Ser Lys Ile Asn Lys Val Phe Ser Thr Pro Ile Pro Phe Ser Tyr Ser
 850 855 860
 Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile
 865 870 875 880
 Leu Lys Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile
 885 890 895
 Ser Asp Ile Ser Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala
 900 905 910
 Gln Leu Val Pro Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn
 915 920 925
 Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp Ile Glu Tyr Asn
 930 935 940
 Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys
 945 950 955 960
 Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile
 965 970 975
 Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser
 980 985 990
 Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys Asp Ser Ala
 995 1000 1005
 Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys Phe
 1010 1015 1020
 Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn
 1025 1030 1035
 Asp Arg Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met
 1040 1045 1050
 Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn
 1055 1060 1065
 Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr
 1070 1075 1080
 Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro
 1085 1090 1095
 Lys Glu Ile Glu Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe
 1100 1105 1110
 Leu Arg Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr Glu Tyr
 1115 1120 1125
 Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val Gln Leu Lys
 1130 1135 1140
 Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro Ser Tyr Thr
 1145 1150 1155
 Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg Leu Tyr Asn Gly Leu
 1160 1165 1170
 Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn Glu Ile Asp Ser
 1175 1180 1185

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Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val Ser Tyr Asn
1190                                     1195                               1200

Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn Ala Phe
1205                                     1210                               1215

Asn Asn Leu Asp Arg Ile Leu Leu Val Gly Tyr Asn Ala Pro Gly
1220                                     1225                               1230

Ile Pro Leu Tyr Lys Lys Met Glu Ala Val Lys Leu Arg Asp Leu
1235                                     1240                               1245

Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp Lys Asn Ala
1250                                     1255                               1260

Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp
1265                                     1270                               1275

Pro Asn Arg Asp Ile Leu Ile Ala Ser Asn Ala Tyr Phe Asn His
1280                                     1285                               1290

Leu Lys Asp Lys Ile Leu Gly Cys Asp Trp Tyr Phe Val Pro Thr
1295                                     1300                               1305

Asp Glu Gly Trp Thr Asn Asp
1310                                     1315

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<210> SEQ ID NO 5
<211> LENGTH: 1315
<212> TYPE: PRT
<213> ORGANISM: Clostridium tetani

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<400> SEQUENCE: 5

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Met Pro Ile Thr Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn Asn
1      5      10      15

Asp Thr Ile Ile Met Met Glu Pro Pro Tyr Cys Lys Gly Leu Asp Ile
20      25      30

Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Val Pro Glu
35      40      45

Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn Pro Pro Ser Ser
50      55      60

Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro Asn Tyr Leu Arg Thr
65      70      75      80

Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr Met Val Lys Leu Phe Asn
85      90      95

Arg Ile Lys Asn Asn Val Ala Gly Glu Ala Leu Leu Asp Lys Ile Ile
100     105     110

Asn Ala Ile Pro Tyr Leu Gly Asn Ser Tyr Ser Leu Leu Asp Lys Phe
115     120     125

Asp Thr Asn Ser Asn Ser Val Ser Phe Asn Leu Leu Glu Gln Asp Pro
130     135     140

Ser Gly Ala Thr Thr Lys Ser Ala Met Leu Thr Asn Leu Ile Ile Phe
145     150     155     160

Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu
165     170     175

Arg Val Asp Asn Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser
180     185     190

Ile Met Gln Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn
195     200     205

Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe
210     215     220

Gln Asp Pro Ala Leu Leu Leu Met His Gln Leu Ile His Val Leu His
225     230     235     240

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Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys
 245 250 255

Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu
 260 265 270

Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile Asp Ile Lys
 275 280 285

Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys Ala Ile Ala Asn
 290 295 300

Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp
 305 310 315 320

Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser
 325 330 335

Asn Gly Gln Tyr Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn
 340 345 350

Ser Ile Met Tyr Gly Phe Thr Glu Ile Glu Leu Gly Lys Lys Phe Asn
 355 360 365

Ile Lys Thr Ala Leu Ser Phe Phe Ser Met Asn His Asp Pro Val Lys
 370 375 380

Ile Pro Asn Leu Leu Asp Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe
 385 390 395 400

Asn Ile Glu Ser Lys Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met
 405 410 415

Arg Val Asn Thr Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val
 420 425 430

Ser Lys Leu Ile Gly Leu Cys Lys Lys Ile Ile Pro Pro Thr Asn Ile
 435 440 445

Arg Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly
 450 455 460

Glu Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu
 465 470 475 480

Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser Tyr
 485 490 495

Asn Thr Lys Asn Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp Lys Ile
 500 505 510

Ile Val Asp Tyr Asn Leu Gln Ser Lys Ile Thr Leu Pro Asn Asp Arg
 515 520 525

Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro Glu Tyr Lys Ser
 530 535 540

Asn Ala Ala Ser Thr Ile Glu Ile His Asn Ile Asp Asp Asn Thr Ile
 545 550 555 560

Tyr Gln Tyr Leu Tyr Ala Gln Lys Ser Pro Thr Thr Leu Gln Arg Ile
 565 570 575

Thr Met Thr Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile
 580 585 590

Tyr Ser Tyr Phe Pro Ser Val Ile Ser Lys Val Asn Gln Gly Ala Gln
 595 600 605

Gly Ile Leu Phe Leu Gln Trp Val Arg Asp Ile Ile Asp Asp Phe Thr
 610 615 620

Asn Glu Ser Ser Gln Lys Thr Thr Ile Asp Lys Ile Ser Asp Val Ser
 625 630 635 640

Thr Ile Val Pro Tyr Ile Gly Pro Ala Leu Asn Ile Val Lys Gln Gly
 645 650 655

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Tyr Glu Gly Asn Phe Ile Gly Ala Leu Glu Thr Thr Gly Val Val Leu
 660 665 670
 Leu Leu Glu Tyr Ile Pro Glu Ile Thr Leu Pro Val Ile Ala Ala Leu
 675 680 685
 Ser Ile Ala Glu Ser Ser Thr Gln Lys Glu Lys Ile Ile Lys Thr Ile
 690 695 700
 Asp Asn Phe Leu Glu Lys Arg Tyr Glu Lys Trp Ile Glu Val Tyr Lys
 705 710 715 720
 Leu Val Lys Ala Lys Trp Leu Gly Thr Val Asn Thr Gln Phe Gln Lys
 725 730 735
 Arg Ser Tyr Gln Met Tyr Arg Ser Leu Glu Tyr Gln Val Asp Ala Ile
 740 745 750
 Lys Lys Ile Ile Asp Tyr Glu Tyr Lys Ile Tyr Ser Gly Pro Asp Ala
 755 760 765
 Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn Lys Leu Glu Glu
 770 775 780
 Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser
 785 790 795 800
 Ser Arg Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln
 805 810 815
 Leu Leu Glu Phe Asp Thr Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile
 820 825 830
 Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Lys Lys Leu Glu
 835 840 845
 Ser Lys Ile Asn Lys Val Phe Ser Thr Pro Ile Pro Phe Ser Tyr Ser
 850 855 860
 Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile
 865 870 875 880
 Leu Lys Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile
 885 890 895
 Ser Asp Ile Ser Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala
 900 905 910
 Gln Leu Val Pro Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn
 915 920 925
 Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp Ile Glu Tyr Asn
 930 935 940
 Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys
 945 950 955 960
 Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile
 965 970 975
 Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser
 980 985 990
 Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys Asp Ser Ala
 995 1000 1005
 Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys Phe
 1010 1015 1020
 Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn
 1025 1030 1035
 Asp Arg Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met
 1040 1045 1050
 Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn
 1055 1060 1065
 Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr

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1070	1075	1080
Val Ser Ile Asp Lys Phe Arg	Ile Phe Cys Lys Ala	Leu Asn Pro
1085	1090	1095
Lys Glu Ile Glu Lys Leu Tyr	Thr Ser Tyr Leu Ser	Ile Thr Phe
1100	1105	1110
Leu Arg Asp Phe Trp Gly Asn	Pro Leu Arg Tyr Asp	Thr Glu Tyr
1115	1120	1125
Tyr Leu Ile Pro Val Ala Ser	Ser Ser Lys Asp Val	Gln Leu Lys
1130	1135	1140
Asn Ile Thr Asp Tyr Met Tyr	Leu Thr Asn Ala Pro	Ser Tyr Thr
1145	1150	1155
Asn Gly Lys Leu Asn Ile Tyr	Tyr Arg Arg Leu Tyr	Asn Gly Leu
1160	1165	1170
Lys Phe Ile Ile Lys Arg Tyr	Thr Pro Asn Asn Glu	Ile Asp Ser
1175	1180	1185
Phe Val Lys Ser Gly Asp Phe	Ile Lys Leu Tyr Val	Ser Tyr Asn
1190	1195	1200
Asn Asn Glu His Ile Val Gly	Tyr Pro Lys Asp Gly	Asn Ala Phe
1205	1210	1215
Asn Asn Leu Asp Arg Ile Leu	Leu Val Gly Tyr Asn	Ala Pro Gly
1220	1225	1230
Ile Pro Leu Tyr Lys Lys Met	Glu Ala Val Lys Leu	Arg Asp Leu
1235	1240	1245
Lys Thr Tyr Ser Val Gln Leu	Lys Leu Tyr Asp Asp	Lys Asn Ala
1250	1255	1260
Ser Leu Gly Leu Val Gly Thr	His Asn Gly Gln Ile	Gly Asn Asp
1265	1270	1275
Pro Asn Arg Asp Ile Leu Ile	Ala Ser Asn Ala Tyr	Phe Asn His
1280	1285	1290
Leu Lys Asp Lys Ile Leu Gly	Cys Asp Trp Tyr Phe	Val Pro Thr
1295	1300	1305
Asp Glu Gly Trp Thr Asn Asp		
1310	1315	

<210> SEQ ID NO 6
 <211> LENGTH: 1315
 <212> TYPE: PRT
 <213> ORGANISM: Clostridium tetani

<400> SEQUENCE: 6

Met Pro Ile Thr	Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn Asn
1	5 10 15
Asp Thr Ile Ile Met Met Glu Pro Pro Ala Cys Lys Gly Leu Asp Ile	
	20 25 30
Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Val Pro Glu	
	35 40 45
Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn Pro Pro Ser Ser	
	50 55 60
Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro Asn Tyr Leu Arg Thr	
	65 70 75 80
Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr Met Val Lys Leu Phe Asn	
	85 90 95
Arg Ile Lys Asn Asn Val Ala Gly Glu Ala Leu Leu Asp Lys Ile Ile	
	100 105 110

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Asn Ala Ile Pro Tyr Leu Gly Asn Ser Tyr Ser Leu Leu Asp Lys Phe
 115 120 125
 Asp Thr Asn Ser Asn Ser Val Ser Phe Asn Leu Leu Glu Gln Asp Pro
 130 135 140
 Ser Gly Ala Thr Thr Lys Ser Ala Met Leu Thr Asn Leu Ile Ile Phe
 145 150 155 160
 Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu
 165 170 175
 Arg Val Asp Asn Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser
 180 185 190
 Ile Met Gln Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn
 195 200
 Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe
 210 215 220
 Gln Asp Pro Ala Leu Leu Leu Met His Gln Leu Ile His Val Leu His
 225 230 235 240
 Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys
 245 250 255
 Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu
 260 265 270
 Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile Asp Ile Lys
 275 280 285
 Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys Ala Ile Ala Asn
 290 295 300
 Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp
 305 310 315 320
 Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser
 325 330 335
 Asn Gly Gln Tyr Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn
 340 345 350
 Ser Ile Met Tyr Gly Phe Thr Glu Ile Glu Leu Gly Lys Lys Phe Asn
 355 360 365
 Ile Lys Thr Ala Leu Ser Phe Phe Ser Met Asn His Asp Pro Val Lys
 370 375 380
 Ile Pro Asn Leu Leu Asp Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe
 385 390 395 400
 Asn Ile Glu Ser Lys Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met
 405 410 415
 Arg Val Asn Thr Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val
 420 425 430
 Ser Lys Leu Ile Gly Leu Cys Lys Lys Ile Ile Pro Pro Thr Asn Ile
 435 440 445
 Arg Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly
 450 455 460
 Glu Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu
 465 470 475 480
 Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser Tyr
 485 490 495
 Asn Thr Lys Asn Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp Lys Ile
 500 505 510
 Ile Val Asp Tyr Asn Leu Gln Ser Lys Ile Thr Leu Pro Asn Asp Arg
 515 520 525
 Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro Glu Tyr Lys Ser

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530			535			540									
Asn	Ala	Ala	Ser	Thr	Ile	Glu	Ile	His	Asn	Ile	Asp	Asp	Asn	Thr	Ile
545					550					555				560	
Tyr	Gln	Tyr	Leu	Tyr	Ala	Gln	Lys	Ser	Pro	Thr	Thr	Leu	Gln	Arg	Ile
				565					570					575	
Thr	Met	Thr	Asn	Ser	Val	Asp	Asp	Ala	Leu	Ile	Asn	Ser	Thr	Lys	Ile
			580					585					590		
Tyr	Ser	Tyr	Phe	Pro	Ser	Val	Ile	Ser	Lys	Val	Asn	Gln	Gly	Ala	Gln
		595					600					605			
Gly	Ile	Leu	Phe	Leu	Gln	Trp	Val	Arg	Asp	Ile	Ile	Asp	Asp	Phe	Thr
	610						615				620				
Asn	Glu	Ser	Ser	Gln	Lys	Thr	Thr	Ile	Asp	Lys	Ile	Ser	Asp	Val	Ser
625					630					635				640	
Thr	Ile	Val	Pro	Tyr	Ile	Gly	Pro	Ala	Leu	Asn	Ile	Val	Lys	Gln	Gly
				645					650					655	
Tyr	Glu	Gly	Asn	Phe	Ile	Gly	Ala	Leu	Glu	Thr	Thr	Gly	Val	Val	Leu
			660					665					670		
Leu	Leu	Glu	Tyr	Ile	Pro	Glu	Ile	Thr	Leu	Pro	Val	Ile	Ala	Ala	Leu
		675					680					685			
Ser	Ile	Ala	Glu	Ser	Ser	Thr	Gln	Lys	Glu	Lys	Ile	Ile	Lys	Thr	Ile
	690						695				700				
Asp	Asn	Phe	Leu	Glu	Lys	Arg	Tyr	Glu	Lys	Trp	Ile	Glu	Val	Tyr	Lys
705					710					715					720
Leu	Val	Lys	Ala	Lys	Trp	Leu	Gly	Thr	Val	Asn	Thr	Gln	Phe	Gln	Lys
				725					730					735	
Arg	Ser	Tyr	Gln	Met	Tyr	Arg	Ser	Leu	Glu	Tyr	Gln	Val	Asp	Ala	Ile
			740					745					750		
Lys	Lys	Ile	Ile	Asp	Tyr	Glu	Tyr	Lys	Ile	Tyr	Ser	Gly	Pro	Asp	Ala
		755					760						765		
Glu	Gln	Ile	Ala	Asp	Glu	Ile	Asn	Asn	Leu	Lys	Asn	Lys	Leu	Glu	Glu
		770					775				780				
Lys	Ala	Asn	Lys	Ala	Met	Ile	Asn	Ile	Asn	Ile	Phe	Met	Arg	Glu	Ser
785					790					795				800	
Ser	Arg	Ser	Phe	Leu	Val	Asn	Gln	Met	Ile	Asn	Glu	Ala	Lys	Lys	Gln
			805						810					815	
Leu	Leu	Glu	Phe	Asp	Thr	Gln	Ser	Lys	Asn	Ile	Leu	Met	Gln	Tyr	Ile
			820					825					830		
Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly	Ile	Thr	Glu	Leu	Lys	Lys	Leu	Glu
		835					840						845		
Ser	Lys	Ile	Asn	Lys	Val	Phe	Ser	Thr	Pro	Ile	Pro	Phe	Ser	Tyr	Ser
		850					855				860				
Lys	Asn	Leu	Asp	Cys	Trp	Val	Asp	Asn	Glu	Glu	Asp	Ile	Asp	Val	Ile
865					870					875				880	
Leu	Lys	Lys	Ser	Thr	Ile	Leu	Asn	Leu	Asp	Ile	Asn	Asn	Asp	Ile	Ile
				885					890					895	
Ser	Asp	Ile	Ser	Gly	Phe	Asn	Ser	Ser	Val	Ile	Thr	Tyr	Pro	Asp	Ala
			900					905					910		
Gln	Leu	Val	Pro	Gly	Ile	Asn	Gly	Lys	Ala	Ile	His	Leu	Val	Asn	Asn
		915					920						925		
Glu	Ser	Ser	Glu	Val	Ile	Val	His	Lys	Ala	Met	Asp	Ile	Glu	Tyr	Asn
		930					935				940				
Asp	Met	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg	Val	Pro	Lys
945					950					955				960	

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Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile
 965 970 975
 Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser
 980 985 990
 Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys Asp Ser Ala
 995 1000 1005
 Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys Phe
 1010 1015 1020
 Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn
 1025 1030 1035
 Asp Arg Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met
 1040 1045 1050
 Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn
 1055 1060 1065
 Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr
 1070 1075 1080
 Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro
 1085 1090 1095
 Lys Glu Ile Glu Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe
 1100 1105 1110
 Leu Arg Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr Glu Tyr
 1115 1120 1125
 Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val Gln Leu Lys
 1130 1135 1140
 Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro Ser Tyr Thr
 1145 1150 1155
 Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg Leu Tyr Asn Gly Leu
 1160 1165 1170
 Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn Glu Ile Asp Ser
 1175 1180 1185
 Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val Ser Tyr Asn
 1190 1195 1200
 Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn Ala Phe
 1205 1210 1215
 Asn Asn Leu Asp Arg Ile Leu Leu Val Gly Tyr Asn Ala Pro Gly
 1220 1225 1230
 Ile Pro Leu Tyr Lys Lys Met Glu Ala Val Lys Leu Arg Asp Leu
 1235 1240 1245
 Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp Lys Asn Ala
 1250 1255 1260
 Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp
 1265 1270 1275
 Pro Asn Arg Asp Ile Leu Ile Ala Ser Asn Ala Tyr Phe Asn His
 1280 1285 1290
 Leu Lys Asp Lys Ile Leu Gly Cys Asp Trp Tyr Phe Val Pro Thr
 1295 1300 1305
 Asp Glu Gly Trp Thr Asn Asp
 1310 1315

<210> SEQ ID NO 7

<211> LENGTH: 1315

<212> TYPE: PRT

<213> ORGANISM: Clostridium tetani

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<400> SEQUENCE: 7

Met Pro Ile Thr Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn Asn
 1 5 10 15
 Asp Thr Ile Ile Met Met Glu Pro Pro Ala Cys Lys Gly Leu Asp Ile
 20 25 30
 Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Val Pro Glu
 35 40 45
 Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn Pro Pro Ser Ser
 50 55 60
 Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro Asn Tyr Leu Arg Thr
 65 70 75 80
 Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr Met Val Lys Leu Phe Asn
 85 90 95
 Arg Ile Lys Asn Asn Val Ala Gly Glu Ala Leu Leu Asp Lys Ile Ile
 100 105 110
 Asn Ala Ile Pro Tyr Leu Gly Asn Ser Tyr Ser Leu Leu Asp Lys Phe
 115 120 125
 Asp Thr Asn Ser Asn Ser Val Ser Phe Asn Leu Leu Glu Gln Asp Pro
 130 135 140
 Ser Gly Ala Thr Thr Lys Ser Ala Met Leu Thr Asn Leu Ile Ile Phe
 145 150 155 160
 Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu
 165 170 175
 Arg Val Asp Asn Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser
 180 185 190
 Ile Met Gln Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn
 195 200 205
 Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe
 210 215 220
 Gln Asp Pro Ala Leu Leu Lys Met His Gln Leu Ile His Val Leu His
 225 230 235 240
 Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys
 245 250 255
 Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu
 260 265 270
 Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile Asp Ile Lys
 275 280 285
 Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys Ala Ile Ala Asn
 290 295 300
 Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp
 305 310 315 320
 Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser
 325 330 335
 Asn Gly Gln Tyr Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn
 340 345 350
 Ser Ile Met Tyr Gly Phe Thr Glu Ile Glu Leu Gly Lys Lys Phe Asn
 355 360 365
 Ile Lys Thr Ala Leu Ser Phe Phe Ser Met Asn His Asp Pro Val Lys
 370 375 380
 Ile Pro Asn Leu Leu Asp Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe
 385 390 395 400
 Asn Ile Glu Ser Lys Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met
 405 410 415

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Arg Val Asn Thr Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val
 420 425 430
 Ser Lys Leu Ile Gly Leu Cys Lys Lys Ile Ile Pro Pro Thr Asn Ile
 435 440 445
 Arg Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly
 450 455 460
 Glu Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu
 465 470 475 480
 Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser Tyr
 485 490 495
 Asn Thr Lys Asn Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp Lys Ile
 500 505 510
 Ile Val Asp Tyr Asn Leu Gln Ser Lys Ile Thr Leu Pro Asn Asp Arg
 515 520 525
 Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro Glu Tyr Lys Ser
 530 535 540
 Asn Ala Ala Ser Thr Ile Glu Ile His Asn Ile Asp Asp Asn Thr Ile
 545 550 555 560
 Tyr Gln Tyr Leu Tyr Ala Gln Lys Ser Pro Thr Thr Leu Gln Arg Ile
 565 570 575
 Thr Met Thr Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile
 580 585 590
 Tyr Ser Tyr Phe Pro Ser Val Ile Ser Lys Val Asn Gln Gly Ala Gln
 595 600 605
 Gly Ile Leu Phe Leu Gln Trp Val Arg Asp Ile Ile Asp Asp Phe Thr
 610 615 620
 Asn Glu Ser Ser Gln Lys Thr Thr Ile Asp Lys Ile Ser Asp Val Ser
 625 630 635 640
 Thr Ile Val Pro Tyr Ile Gly Pro Ala Leu Asn Ile Val Lys Gln Gly
 645 650 655
 Tyr Glu Gly Asn Phe Ile Gly Ala Leu Glu Thr Thr Gly Val Val Leu
 660 665 670
 Leu Leu Glu Tyr Ile Pro Glu Ile Thr Leu Pro Val Ile Ala Ala Leu
 675 680 685
 Ser Ile Ala Glu Ser Ser Thr Gln Lys Glu Lys Ile Ile Lys Thr Ile
 690 695 700
 Asp Asn Phe Leu Glu Lys Arg Tyr Glu Lys Trp Ile Glu Val Tyr Lys
 705 710 715 720
 Leu Val Lys Ala Lys Trp Leu Gly Thr Val Asn Thr Gln Phe Gln Lys
 725 730 735
 Arg Ser Tyr Gln Met Tyr Arg Ser Leu Glu Tyr Gln Val Asp Ala Ile
 740 745 750
 Lys Lys Ile Ile Asp Tyr Glu Tyr Lys Ile Tyr Ser Gly Pro Asp Ala
 755 760 765
 Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn Lys Leu Glu Glu
 770 775 780
 Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser
 785 790 795 800
 Ser Arg Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln
 805 810 815
 Leu Leu Glu Phe Asp Thr Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile
 820 825 830

