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(54) Title: MATERIALS AND METHODS FOR PRODUCING A BIOLOGICAL TOXIN

(57) Abstract: The current invention provides a method of producing a heterologous biological toxin or subunit thereof in a bacterium, such as a non-pathogenic bacterium. The current invention also provides a method of producing botulinum neurotoxin in a bacterium other than *Clostridium spp.* The current invention further provides a method of producing botulinum neurotoxin in *Lactococcus lactis*. Furthermore, the current invention provides a hybrid toxin comprising a first subunit of a first biological toxin and a carrier which may be a second subunit from a second biological toxin. Additionally, the current invention provides a method of treating a disease in a subject by administering a pharmaceutically effective amount of a hybrid toxin to the subject, wherein the hybrid toxin is specifically designed for the treatment of the disease in the subject.



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DESCRIPTION

MATERIALS AND METHODS FOR PRODUCING A BIOLOGICAL TOXIN

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CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of U.S. Provisional Application Serial No. 61/831,920, filed June 6, 2013, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

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BACKGROUND OF THE INVENTION

Biological toxins require strict safeguards against exposure through inhalation, absorption through skin or mucous membranes, ingestion, or percutaneous injury because these toxins are extremely hazardous, even in minute quantities. However, appropriate use of biological toxins provides medical treatment options for various disorders.

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For example, over the past three decades, there has been a continuing fascination with botulinum neurotoxin (BoNT) and the opportunities for use of this potentially deadly neurotoxin as a treatment in certain diseases. BoNT interferes with the release of acetylcholine from presynaptic nerve endings and prevents muscle contractions and/or glandular secretion. Since BoNT is used primarily as a focal injectable treatment, resulting in fewer side effects than systemically administered medications, it has evolved to become the treatment of choice for management of many forms of dystonia, limb spasticity, cosmetic glabellar lines, hyperhidrosis, and sialorrhea [1]. The use of OnabotulinumtoxinA (Botox Cosmetic) to address migraines was approved by the FDA in 2010 [2], and its use to treat bladder hyperactivity was approved in 2011 [3–5]. BoNT also has a broad spectrum of off-label indications, including focal hand dystonia, lower limb spasticity [6,7], management of chronic anal fissures [4], and tension headaches [8]. The commercially available neurotoxins include three main brands of BoNT/A: OnabotulinumtoxinA (BOTOX®), AbobotulinumtoxinA (DYSPORT®), IncobotulinumtoxinA (XEOMIN®), and also a brand of BoNT type B, RimabotulinumtoxinB (MYOBLOC® or NEUROBLOC®) [9]. Each formulation has

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specific clinical recommendations which have been formulated based on the available science, and on differing biochemical profiles.

An issue with production of BoNT has been the use the pathogenic *Clostridium spp.* to manufacture the drug. The process is costly, labor intensive, requires special equipment, and is extremely hazardous, particularly in view of the fact that disinfection includes procedures to destroy the *Clostridium* spores. While production of other biological toxins may present similar problems with pathogenicity and there are always safety concerns in manufacturing biological toxins, working with spores is particularly problematic. Therefore, a safer method of manufacturing biological toxins in non-pathogenic bacteria is desirable.

BRIEF SUMMARY OF THE INVENTION

The current invention provides a method of producing a biological toxin in non-pathogenic bacteria. The current invention also provides a method of producing botulinum neurotoxin (BoNT) in a bacterium other than *Clostridium spp.* A further aspect of the invention provides a method of producing a biological toxin in a lactic acid bacterium (LAB), such as *Lactococcus lactis*.

The current invention also provides a method of producing hybrid toxins (also referred to herein as chimeric toxins or fusion proteins), wherein the hybrid toxins comprise multiple subunits from different biological toxins. An example of a hybrid toxin of the current invention comprises a first subunit from a first biological toxin and a carrier, such as a second subunit from a second biological toxin.

Furthermore, another aspect of the invention provides a method of treating a disorder in a subject in need thereof by administering an effective amount of a biological toxin to the subject. Another aspect of the invention provides a method of treating a disease in a subject by administering an effective amount of a hybrid toxin comprising multiple subunits to the subject, wherein the multiple subunits of the hybrid toxin are selected to treat the disease in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Cloning of light chain (Lc) and (Hc) of BoNT in the expression vectors for expression of BoNT in *Lactococcus lactis*.

Figure 2. Schematic representation of a hybrid toxin comprising a catalytic Lc (payload) subunit and a targeting Hc (carrier).

DETAILED DESCRIPTION OF THE INVENTION

5 The correct processing of biological toxins by the toxin producing cell is believed to be critical for obtaining biologically active toxin. For example, stringent need for correct processing of the holotoxin, operated by specific proteases, led experts to believe that functional botulinum neurotoxin (BoNT) can only be produced in *Clostridium botulinum* or the closely related *Clostridium argentinense*. The current invention
10 provides a method of producing biological toxins, for example, BoNT, in a non-pathogenic host, thereby greatly improving the safety associated with manufacturing hazardous toxins using pathogenic bacteria and dramatically decreasing the costs of both equipment and personnel. The current invention also provides a method of producing BoNT in a bacterium other than *Clostridium spp.*

15 One aspect of the current invention is a method of producing a biological toxin, or a subunit thereof, using a bacterium other than *Clostridium botulinum*. The method of the current invention comprises:

 a) introducing in to the bacterium an expressible nucleic acid encoding the biological toxin or subunit; and

20 b) culturing the bacterium containing the expressible nucleic acid encoding the biological toxin or subunit under the conditions that allow expression of the biological toxin.

 Optionally, the method further comprises c) purifying the biological toxin or subunit from the culture of the bacterium.

25 Preferably, a non-pathogenic bacterium is used; however, pathogenic bacterium may be desired in some circumstances.

 As used herein, the term “biological toxin” refers to a poisonous biomolecule, for example a protein, which is produced by living cells or organisms and is capable of causing a disorder when introduced into the body of an animal, for example, a human.

30 The source for the biological toxin can be any organism, for example, plants, animals, bacteria, fungi, protists, lower eukaryotes, and higher eukaryotes. In some embodiments, the biological toxin is a “wild-type” toxin, such as botulinum toxin, or other toxin

produced by a bacterium other than *Clostridium botulinum*. (e.g., a non-pathogenic bacterium such as a lactic acid bacteria or a *Bacillus*), wherein the toxin is heterologous to the non-*Clostridium* bacterium. In some embodiments, the biological toxin is a hybrid toxin, representing a genetic fusion of one or more biological toxins or one or more subunits thereof (a payload) and one or more carriers. The hybrid toxins may be produced using *Clostridium spp.* (e.g., *Clostridium botulinum*) or a bacterium other than *Clostridium botulinum* (e.g., a non-pathogenic bacterium such as a lactic acid bacteria or a *Bacillus*). In some embodiments, the biological toxin comprises one or more serotypes of botulinum toxin A-G.

As used herein, the term “carrier” refers to a molecule that targets a “payload” molecule such as a biological toxin, to a target cell or cell type. The carrier may be homologous (natural) to the payload or heterologous to the payload. Typically, the target cell is not a natural target cell. In some embodiments, the carrier is a subunit of a second biological toxin. In some embodiments, the first subunit of the first biological toxin is the active subunit of the first biological toxin and the carrier is a targeting subunit of a second biological toxin. In some embodiments, the first subunit of the first biological toxin is the active subunit of botulinum neurotoxin and the carrier is selected from among a targeting subunit of Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

Through genetic fusion, one or more carriers and one or more payloads can be arranged at any location along the length of the chimeric construct and oriented with respect to one another. For example, the fusion protein of carrier and payload can comprise the carrier on the N-terminus and the payload on the C-terminus of the fusion protein (carrier-payload), or the payload on the N-terminus and the carrier on the C-terminus of the fusion protein (payload-carrier). The arrangement of the domains (payload and carrier) can be controlled by their arrangement in the encoding nucleic acid molecules.

Optionally, a cleavable linker can be inserted between the payload and the carrier. An example of a cleavable linker is the target polypeptide of the BoNT light chain itself [13]. A cleavable linker can be cleaved under the conditions for which it is designed and/or utilized. For example, a cleavable linker can be cleaved by an enzyme endogenous

to the subject or by an exogenous enzyme that is administered to the subject before, during, or after administration of the toxin.

In another embodiment, a linker that is resistant to cleavage can be genetically inserted between the payload and the carrier. For example, a cleavage resistant SNAP25-
5 BoNT light chain cleavable linker or a mutant SNAP25 which is active yet resistant to cleavage may be utilized, as identified in [14]. As used herein, term “heterologous” in connection with a biological toxin or subunit thereof means the toxin or subunit is a type not normally produced by that bacteria. In the context of a carrier, a carrier is heterologous to its payload if the carrier and payload do not naturally occur within the
10 same molecule.

As used herein, the term “hybrid toxin” refers to a toxin comprising multiple (two or more) subunits, wherein the subunits originate in different biological toxins and are not naturally present together in a biological toxin. For example, in some embodiments, a hybrid toxin of the current invention comprises the active subunit of BoNT and targeting
15 subunit of Tetanus toxin.

As used herein, the term “non-pathogenic bacterium” means a bacterium that is generally not capable of causing a disease in human or non-human animal. It should be understood, however, that a non-pathogenic bacterium of the invention can become an opportunistic pathogen under some circumstances and occasionally cause a disease, for
20 example, in an immuno-compromised animal.

The bacterium may be cultured in a culture vessel (dish, plate, bottle, or other suitable substrate) of any scale (large or small), as an open or closed culture. In some embodiments, the bacterium is cultured in a bioreactor, such as a batch, fed-batch, or continuous bioreactor.

25 The biological toxin produced by the method of the current invention can comprise one or more subunits. An example of the biological toxin that can be produced by the method of the current invention is botulinum neurotoxin.

Methods of purifying a polypeptide expressed in bacteria are well known to a person of ordinary skill in the art and these methods are within the purview of the current
30 invention.

Many biological toxins follow a multi-subunit configuration, for example, the AB configuration, where A subunit is the catalytic subunit exerting the cellular damage, while

B subunit targets the holotoxin to specific sites in host tissues. B subunit can also act as a chaperone in some cases [10, 11]. Such biological toxins are called binary toxins. Examples of such binary biological toxins are BoNT, Anthrax toxin, Tetanus toxin, ricin, Shiga-like toxin, Shiga toxin, *etc.* Additional binary toxins are well known to a person of ordinary skill in the art and are within the purview of the current invention. Typically, the biological activity of the A subunit is 5-6 logs lower when it acts alone compared to when it acts in concert with the targeting subunit. For example, BoNT serotypes A-G follow this design [12]. BoNT A-G are produced as 150KDa inactive holopeptides that undergo enzymatic cleavage thereby removing a short peptide, and thus releasing an active toxin composed of a 50KDa catalytic light chain (Lc) and a 100KDa heavy chain targeting moiety (Hc). Lc and Hc remain in contact through a disulfide bridge.

An aspect of the current invention also provides a method of producing separate subunits of a biological toxin using two or more bacteria, wherein the biological toxin comprises multiple subunits. In another embodiment, the current invention provides a method of producing a biological toxin comprising a first subunit and a carrier, such as a second subunit. The method of producing a biological toxin comprising a first subunit and a carrier according to the current invention, comprises:

a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit;

b) introducing into a second bacterium an expressible nucleic acid encoding the carrier (*e.g.*, second subunit); and

c) culturing the first and the second bacteria containing the expressible nucleic acid encoding the first subunit and the carrier under conditions that allow expression of the first subunit and the carrier.

Optionally, the method further comprises: d) purifying the first subunit and the carrier from the culture of the first bacterium and the second bacterium. Optionally, the method further comprises: e) assembling the first subunit and the carrier to produce the biological toxin.

The first bacterium and the second bacterium can be cultured in the same culture vessel (*i.e.* co-cultured). Alternately, the first and the second bacteria can be cultured in separate culture vessels. For example, the first non-pathogenic bacterium may be

cultured in a first bioreactor and the second bacterium may be cultured in a second bioreactor.

In an embodiment of the invention, the first bacterium and the second bacterium belong to the same species and strain. For example, the bacterium used to manufacture a multisubunit biological toxin is *Lactococcus lactis*.

Alternately, the first bacterium and the second bacterium can belong to different species and strains. When the first bacterium and the second bacterium belong to different species and strain, in one embodiment, the first bacterium belongs to the genus *Lactococcus* and the second bacterium belongs to the genus *Bacillus*. In an embodiment of the invention, the first bacterium is *Lactococcus lactis* and the second bacterium is *Bacillus subtilis*.

Table 1 lists examples of toxins and subunits that can be utilized in producing biological toxins, compositions, microorganisms, and methods of the invention, and corresponding references and GenBank accession numbers, including sequences, are incorporated herein by reference in their entirety.

Table 1. Exemplified Toxins and Subunits

Toxin name	GenBank Protein Accession Number	Reference
Botulinum neurotoxin type A holotoxin from <i>Clostridium botulinum</i>	P10845.4	Thompson, D.E. <i>et al.</i> , "The complete amino acid sequence of the <i>Clostridium botulinum</i> type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene," <i>Eur. J. Biochem.</i> , 189 (1), 73-81 (1990).
Botulinum neurotoxin type A light chain from <i>Clostridium botulinum</i>	AAO21363	Seong, H.Y. <i>et al.</i> , "Effects of minor arginyl and isoleucyl tRNA on the expression of botulinum toxin light chain in <i>Escherichia coli</i> ," Unpublished.
Botulinum neurotoxin type A heavy chain from <i>Clostridium botulinum</i>	Heavy chain of Botulinum neurotoxin Type A comprises amino acids 448-1295 of the holotoxin. The heavy chain comprises a translocation domain and a	Lacy, D.B., <i>et al.</i> , "Crystal structure of botulinum neurotoxin type A and implications for toxicity," <i>Nature Structural Biology</i> , 5, 898 - 902 (1998).

Toxin name	GenBank Protein Accession Number	Reference
	receptor binding domain.	
Botulinum neurotoxin type B holotoxin from <i>Clostridium botulinum</i>	AAA23211	Bodsworth, N.J. <i>et al.</i> , "Complete nucleotide sequence of the <i>Clostridium botulinum</i> gene encoding the type B neurotoxin," unpublished (1993).
Botulinum neurotoxin type B light chain from <i>Clostridium botulinum</i>	AAB22656	Kurazono, H. <i>et al.</i> , "Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A," <i>J. Biol. Chem.</i> , 267 (21), 14721-14729 (1992).
Botulinum neurotoxin type B heavy chain from <i>Clostridium botulinum</i>	Heavy chain of Botulinum neurotoxin type B comprises of amino acids 452-1291 of the holotoxin. The heavy chain comprises a translocation domain and a receptor binding domain.	Kurazono, H. <i>et al.</i> , "Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A," <i>J. Biol. Chem.</i> , 267 (21), 14721-14729 (1992).
Lethal Factor of Anthrax toxin from <i>Bacillus anthracis</i>	P15917	Bragg, T.S., <i>et al.</i> , "Nucleotide sequence and analysis of the lethal factor gene (lef) from <i>Bacillus anthracis</i> ," <i>Gene</i> , 81(1), 45-54 (1989).
Edema Factor of Anthrax toxin from <i>Bacillus anthracis</i>	AAA79215	Robertson, D.L., <i>et al.</i> , "Nucleotide sequence of the <i>Bacillus anthracis</i> edema factor gene (cya): a calmodulin-dependent adenylate cyclase," <i>Gene</i> , 73 (2), 363-371 (1988).
Protective Antigen of Anthrax toxin from <i>Bacillus anthracis</i>	AAF86457	Cohen, S. <i>et al.</i> , "Attenuated nontoxinogenic and nonencapsulated recombinant <i>Bacillus anthracis</i> spore vaccines protect against anthrax," <i>Infect. Immun.</i> , 68 (8), 4549-4558 (2000).
Tetanus toxin from <i>Clostridium tetani</i>	P04958	Eisel, U. <i>et al.</i> , "Tetanus toxin: primary structure, expression in <i>E. coli</i> , and homology with botulinum toxins," <i>EMBO J.</i> , 5 (10), 2495-2502 (1986).
Ricin toxin from <i>Ricinus communis</i> (castor bean)	P02879	Halling, K.C. <i>et al.</i> , "Genomic cloning and characterization of a ricin gene from <i>Ricinus communis</i> ," <i>Nucleic Acids Res.</i> , 13 (22), 8019-8033 (1985).
Shiga toxin A subunit from	CAC05622	Unkmeir, A. <i>et al.</i> , "Structural analysis of phage-borne stx genes and their flanking

Toxin name	GenBank Protein Accession Number	Reference
<i>Shigella dysenteriae</i>		sequences in shiga toxin-producing <i>Escherichia coli</i> and <i>Shigella dysenteriae</i> type 1 strains,” <i>Infect. Immun.</i> , 68(9), 4856-4864 (2000).
Shiga toxin B subunit from <i>Shigella dysenteriae</i>	CAC05623	Unkmeir, A. <i>et al.</i> , “Structural analysis of phage-borne stx genes and their flanking sequences in shiga toxin-producing <i>Escherichia coli</i> and <i>Shigella dysenteriae</i> type 1 strains,” <i>Infect. Immun.</i> , 68(9), 4856-4864 (2000).
Shiga-like toxin type II - A subunit from <i>Escherichia coli</i>	AAA16360	Paton, A.W. <i>et al.</i> , “Polymerase chain reaction amplification, cloning and sequencing of variant <i>Escherichia coli</i> Shiga-like toxin type II operons,” <i>Microb. Pathog.</i> , 15(1), 77-82 (1993).
Shiga-like toxin type II -B subunit from <i>Escherichia coli</i>	CAA85781	Paton, A.W. <i>et al.</i> , “Comparative toxicity and virulence of <i>Escherichia coli</i> clones expressing variant and chimeric Shiga-like toxin type II operons,” <i>Infect. Immun.</i> , 63(7), 2450-2458 (1995).

The amino acid sequences of the toxins and subunits exemplified in Table 1 are provided below.

5 MPFVNKQFNYKDPVNGVDIA YIKIPNVGQM QPVKAFKIH NKIWVIPERDTFTNPE
EGDLNPPPEAKQVPVSYYDSTYLSTDNEKDN YLKGVTKL FERIYSTDLGRMLLTS
IVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVIIGPSADIIQFECKSFG
HEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLL GAGKFATDPAVTLAHEL
IHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHD AKFIDSLQEN
10 EFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDK
LKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGF
NLRNTNLAANFNGQNT EINN MNFTKLKNFTGLFEFYKLLCVRGIITSKTKSLDKG
YNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISLDLIQQ
YYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFHYLRAQE
15 FEHGKSRIALTNSVNEALLNPSRVYTFSSDYVKKV NKATEAAMFLGWVEQLVY
DFTDETSEVSTTDKIADITIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIA
IPVLGTFALVSYIANKVLT VQTIDNALS KRNEKWDEVYKYIVTNWLAKVNTQIDL
IRKKMKEALENQAEATKAIINYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMIN
INKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVDRLK

DKVNNLTLDIPFQLSKYVDNQRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYAS
KINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFN
SISLNNEYTIINCMENNSGWKVS LNYGEIIWTLQDTQEIKQRVVF KYSQMINISDYI
NRWIFVTITNNRLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYI
5 WIKYFNLFDKELNEKEIKDLYDNQSN SGILKDFWGDY LQYDKPYMLNLYDPN
KYVDVNNVGIRGYMYLK GPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIV
RNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKND
QGITNKCKMNLQDNNGNDIGFIGFHQFN NIAKLVASNWYNRQIERSSRTLGC SW
EFIPVDDGWGERPL (Botulinum neurotoxin type A holotoxin from *Clostridium*
10 *botulinum*; Accession no. P10845.4; SEQ ID NO:1)

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQM QVKAFKIH NKI WVIPERDTFTNPEE
GDLNPPPEAKQVPVSYYDSTYLSTDNEKDNYLKG VTKLFERIYSTDLGRMLLTSI
VRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVIIGPSADIIQFECKSFG
15 HEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLL GAGKFATDPAVTLAHEL
IHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQEN
EFRLYYYNKFKDIASTLNKASIVGTTASLQYMKNV FKEKYLLSEDTSGKFSVDKL
KFDKLYKMKTEIYTEDDNFVKFFKVLNRKTYL NFDKAVFKINIVPKVNYTIYDGF
NLRNTNLAANFNGQNT EINN MNFTKLKNFTGLFEFYKLLCVRGIITSKTKSLDEG
20 YNK (Botulinum neurotoxin type A light chain from *Clostridium botulinum*; Accession
no. AAO21363; SEQ ID NO:2)

MPVTINNFNYNDPIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKP
EDFNKSSGIFNRDVCEYYDPDY LNTNDKKNIFLQTMIKLFNRIKSKPLGEKLLEMI
25 INGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNEN
ETIDIGIQNHFASREGFGGIMQM KFCPEYVS VFNNVQENKGASIFNRRGYFSDPAL
ILMHელიHVLHGLYGIKVDDLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPS
TDKSIYDKVLQNFRGIVDRLNKVLVCISDPNININ IYKNKFKDKYKFVEDSEGKYS
IDVESFDKLYKSLMFGFTETNIAENYKIKTRASYFSDSLPPVKIKNLLDNEIYTIEE
30 GFNISDKDMEKEYRGQNKAINKQAYEEISKEHLAVYKIQMCKSVKAPGICIDVDN
EDLFFIADKNSFSDDL SKNERIEYNTQSNYIENDFPINELILD TD LISKIELPSENTES
LTDFNVDPVPVYEKQPAIKKIFTDENTIFQYLYSQT FPLDIRDISLTSSFDDALLFSN

KVYSFFSMDYIKTANKVVEAGLFAGWVKQIVNDFVIEANKSNTMDKIADISLIVP
YIGLALNVGNETAAGNFENAFEIAGASILLEFIPELLIPVVGAFLLSEYIDNKNKIIK
TIDNALTNRNEKWSDMYGLIVAQWLSTVNTQFYTIKEGMYKALNYQAQALEEII
KYRYNIYSEKEKSNINIDFNDINSKLNEGINQAIDNINNFININGCSVSYLMKKMIPLA
5 VEKLLDFDNTLKKNLLNYIDENKLYLIGSAEYEKSKVNKYLKTIMPFDLISIYTND
TILIEFMNKYNSEILNNIILNLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLT
SSANSKIRVTQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNN
SGWKISIRGNRIIWTLIDINGKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYI
NGKLESNTDIKDIREVIANGEIIFKLDGDDIDRTQFIWMKYFSIFNTELSQSNIIEERYK
10 IQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSKYNQN
SKYINYRDLYIGEKFIIRKSNSSQSINDDIVRKEDYIYLDFFNLNQEWVRVYTYKYF
KKEEEKLFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFY
ESGIVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE (Botulinum
neurotoxin type B holotoxin from *Clostridium botulinum*; Accession no. AAA23211;
15 SEQ ID NO:3)

MPVTINNFNYNDPIDNNNIIMMEPPFARGTGRIYYKAFKITDRIWIIPERYTFGYKP
EDFNKSSGIFNRDVCEYYDPDYLNTNDKKNIFLQTMIKLFNRIKSKPLGEKLLEMI
INGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNEN
20 ETIDIGIQNHFASREGFGGIMQMKFCPEYVSFVNNVQENKGASIFNRRGYFSDPAL
ILMHELIVLHGLYGIKVDLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPS
TDKSIYDKVLQNFRGIVDRLNKVLVCISDPSININIYKNKFKDKYKFVEDSEGKYSI
DVESFDKLYKSLMFGFTETNIAENYKIKTRASYFSDSLPPVKIKNLLDNEIYTIEEG
FNISDKDMEKEYRGQNKAINQAYEEISKEHLAVYKIQMCKSVKAPGICIDVDN
25 (Botulinum neurotoxin type B light chain from *Clostridium botulinum*; Accession no.
AAB22656; SEQ ID NO:4)

MNIKKEFIKVISMCLVTAITLSGPVFIPLVQGAGGHGDVGMHVKEKEKNKDENK
RKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSPVLEMYKA
30 IGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVYAKEGYEPVLVIQ
SSDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDL
LFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEAFNY
MDKFNEQEINLSLEELKDQRMRLARYEKWEKIKQHYQHWSDSLSEEGRGLLKKL
QIPIEPKKDDIIHSLSQEEKELLKRIQIDSSDFLSTEEKEFLKKLQIDIRDSLSEEEKEL
35 LNRIQVDSSNPLSEKEKEFLKKLKLDIQPYDINQRLQDTGGLIDSPSINLDVRKQY
KRDIQNIDALLHQSIGSTLYNKIYLYENMNINNLTLATLGADLVDSTDNTKINRGIF

NEFKKNFKYSSISNYMIVDINERPALDNERLKWRIQLSPDTRAGYLENGKLILQRN
IGLEIKDVQIIKQSEKEYIRIDAKVVPKSKIDTKIQEAQLNINQEWNKALGLPKYTK
LITFNVHNRYASNIVESAYLILNEWKNNIQSDLIKKVTNYLVDGNGRFVFTDITLP
NIAEQYTHQDEIYEQVHSGGLYVPESRSILLHGPSKGVELRNDSEGFIHEFGHAVD
5 DYAGYLLDKNQSDLVTSKKFIDIFKEEGSNLTSYGRTNEAEFFAEAFRLMHSTD
HAERLKVQKNAPKTFQFINDQIKFIINS (Lethal Factor of Anthrax toxin from *Bacillus*
anthracis; Accession no. P15917; SEQ ID NO:5)

MTRNKFIPNKFISIISFSVLLFAISSSQAIENVAMNEHYTESDIKRNHKTEKNKTEKE
10 KFKDSINNLVKTEFTNETLDKIQQTQDLLKKIPKDVLEIYSELGGEIYFTDIDLVEH
KELQDLSEEEKNSMNSRGEKVPFASRFVFEKKRETPKLIINIKDYAINSEQSKEVY
YEIGKGISLDIISKDKSLDPEFLNLIKSLSDSDSSDLLFSQKFKEKLELNNKSIDINF
IKENLTEFQHAFLAFSYYFAPDHRTVLELYAPDMFEYMNKLEKGGFEKISESLK
KEGVEKDRIDVLKGEKALKASGLVPEHADAFKKIARELNTYILFRPVNKLATNLI
15 KSGVATKGLNEHGKSSDWGPVAGYIPFDQDLSKKHGGQLAVEKGNLENKKSITE
HEGEIGKIPLKLDHLRIEELKENGILKGKKEIDNGKKYYLLESNNQVVEFRISDEN
NEVQYKTKEGKITVLGEKFNWRNIEVMAKNVEGVLKPLTADYDLFALAPSLTEI
KKQIPTKRMDKVVNTPNSLEKQKGVTNLLIKYGIERKPDSTKGTLNWKQKQMLD
RLNEAVKYTGTYGGDVVNHGTEQDNEEFPEKDNEIFIINPEGEFILTKNWEMTGR
20 FIEKNITGKDYLYYFNRSYNKIAPGNKAYIEWTDPITKAKINTIPTSAEFIKNLSSIR
RSSNVGVYKDSGDKDEFKESVKKIAGYLSDYNSANHIFSQEKKRKISIFRGIQ
AYNEIENVLKSQIAPEYKNYFQYLERITNQVQLLLTHQKSNIFFKLLYKQLNFT
ENETDNFEVFQKIIDEK (Edema Factor of Anthrax toxin from *Bacillus anthracis*;
Accession no. AAA79215; SEQ ID NO:6)

25 MKKRKVLIPLMALSTILVSSTGNLEVIQAEVKQENRLLNESESSSQGLLGYFYFSDL
NFQAPMVVTSSTTGDLSSIPSELENIPSENQYFQSAIWSGFIKVKKSDEYTFATSAD
NHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTTEKGLDFKLYWTD
SQNKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYT
30 VDVKNKRFTLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKTGRIDKNVS
PEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSQTRTISKNTSTSRHTHTSEVH
GNAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTAR
LNANIRYVNTGTAPIYNVLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYYPSKN

LAPIALNAQDDFSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIATYNFENGVR
VDTGSNWSEVLPIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEA
LKIAFGFNEPNGNLQYQGKDITEFDNFNDQQT SQNIKNQLAELNATNIYTVLDKIK
LNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLL NIDKDIRKI
5 LSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPY
KVN VYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG (Protective Antigen of
Anthrax toxin from *Bacillus anthracis*; Accession no. AAF86457; SEQ ID NO:7)

MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPE
10 DFNPPSSLIEGASEYYDPNYLR TDS DKDRFLQTMVKLFNRIKNNVAGEALLDKIIN
AIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAML TNLIIFGPGPVLNKN
EVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTSLTIGKSKYFQ
DPALLMHელიHVLHGLYGMQVSSHEIIPSKQEIYMQHTYPISAEELFTFGGQDAN
LISIDIKNDLYEKTLDYKA IANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSN
15 GQYIVNEDKFQILYNSIMYGFTIELGKKFNIKTRLSYFSMNHDPVKIPNLLDDTIY
NDTEGFNIESKDLKSEYKGQNM RVNTNAFRNVDGSLVSKLIGLCKKIIPPTNIRE
NLYNRTASLTDLGGELCIKIKNEDLT FIAEKNSFSEEPFQDEIVSYNTKNKPLNFNY
SLDKIIVDYNLQSKITLPNDRTTPVTKGIPYAPEYKSNAASTIEIHNIDDNTIYQYLY
AQKSPTTLQRITMTNSVDDALINSTKIYSYFPSVISKVNQGAQGILFLQWVRDIIDD
20 FTNESSQKTIDKISDVSTIVPYIGPALNIVKQGYEGNFIGALETTGVVLLLEYIPEIT
LPVIAALSIAESSTQKEKIIKTIDNFLEKRYEKWIEVYKLVKAKWLGT VNTQFQKR
SYQMYRSLEYQVDAIKKIIDYEYKIYSGPDKEQIAD EINN LKNKLEEKANKAMINI
NIFMRESSRSFLVNQMINEAKKQLLEFDTQSKNILMQYIKANSKFIG
ITELKKLESKINKVFSTPIPF SYSKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDI
25 SGFNSSVITYPDAQLVPGINGKAIHLVN NESSEVIVHKAMDIEYNDMFNNFTVSF
WLRVPKVSASHLEQYGTNEYSIISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAG
EVRQITFRDLPDKFNAYLAN KWVFITITNDRLSSANLYINGVLMGSAEITGLGAIR
EDNNITLKLDRCNNNNQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDFWGNP
LRYDTEYYLIPVASSSKDVQLKNITDYM YLTNAPS YTN GKLN IYYRRLYNGLKFI
30 KRYTPNNEIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNAFNNLDRILRVGY NAP
GIPLYKKMEAVKLRLDKTYSVQLKLYDDKNASLGLVGTHNGQIGNDPNRDILIA

SNWYFNHLKDKILGCDWYFVPTDEGWTND (Tetanus toxin from *Clostridium tetani*;
Accession no. P04958; SEQ ID NO:8)

MKPGGNTIVIWMYAVATWLCFGSTSGWSFTLEDNNIFPKQYPIINFTTAGATVQS
5 YTNFIRAVRGRLTTGADV RHEIPVLPNRVGLPINQRFILVELSNHAELSVTLALDV
TNAYVVG YRAGNSAYFFHPDNQEDAEATHLFTDVQNRYTFAFGGNYDRLEQL
AGNLRENIELGNNGPLEEAISALYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGE
MRTRIRYNRRSAPDPSVITLENSWGRLSTAIQESNQGAFA SPIQLQRRNGSKFSVY
DVSILIP IIALMVYRCAPPPSSQFSL LIRPVVPNFNADVCM DPEPIVRIVGRNGLCV
10 DVRDGRFHNGNAIQLWPCKSNTDANQLWTLKRDNTIRSNGKCLTTYGYSPGVY
VMIYDCNTAATDATRWQIWDNGTIINPRSSLVLAATSGNSGTTLTVQTN IYAVSQ
GWLPTNNTQPFVTTIVGLYGLCLQANSGQVWIEDCSSEKAEQQWALYADGSIRP
QQNRDNCLTSDSNIRETVVKILSCGPASSGQRWMFKNDGTILNLYSGLVLDVRAS
DPSLKQIILYPLHGDPNQIWLPLF (Ricin toxin from *Ricinus communis* (castor bean);
15 Accession no. P02879; SEQ ID NO:9)

MKIIIFRVL TFFFVIFSVNVVAKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGT
SLLMIDSGTGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTG FVNRTNNVFYR
FADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSHS
20 GTSLTQSVARAMLRFVTVTAEALRFRQIQRGFR TTLDDLSGRSYVM TAEVDVLT
LNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASRVARMASD
EFPSMCPADGRVRGITHNKILWDSSTLGAILMRRTISS (Shiga toxin A subunit from
Shigella dysenteriae; Accession no. CAC05622; SEQ ID NO:10)

MKKTL LIAASLSFFSASALATPDCVTGKVEYTKYNDDDTFTVKVGDKELFTNRW
25 NLQSLLLSAQITGMTVTIKTNACHNGGGFSEVIFR (Shiga toxin B subunit from
Shigella dysenteriae; Accession no. CAC05623; SEQ ID NO:11)

MKCILFKWVLCLLLGFSSVSYSREFMIDFSTQQSYVSSLNSIRTEISTPLEHISQGTT
30 SVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIEQNNLYVAGFVNTATNTFYRF
SDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSG
NTMTRDASRAVLR FVTVTAEALRFRQIQGEFRQALSETAPVYTMTPEEVDLT LN

WGRISNVLPEFRGEGGVRVGRISFNNISAILGTVAVILNCHHQGARSVRVAVNEEIQ
PECQITGDRPVIRINNTLWESNTAAAFNRRRAHSLNTSGE

(Shiga-like toxin type II - A subunit from *Escherichia coli*; Accession no. AAA16360;
SEQ ID NO:12)

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MKKMFMAVLFLVSVNAMAADCAKKGKIEFSKYNEDDTFTVKVDGKEYWTSRW
NLQPLLQSAQLTGMTVTIKSSTCESGSGFAEVQFNND

(Shiga-like toxin type II -B subunit from *Escherichia coli*; Accession No. CAA85781;
SEQ ID NO:13)

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In some embodiments, the toxin or toxin subunit includes the initiation methionine, whereas in other embodiments, it does not. For example, the proteins shown above include the initiation methionine, but the invention nevertheless includes those proteins that lack the initiation methionine. This can also be true for the other proteins of different types, different subtypes, and even the different species described elsewhere in the specification.

15

In some embodiments the toxin or toxin subunit comprises or consists of one or more toxins or toxin subunits listed in Table 1. In some embodiments the toxin or toxin subunit comprises or consists of one or more polypeptides selected from among SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, or SEQ ID NO:13.

20

Functional variants and functional fragments of biological toxins or toxin subunits, and nucleic acids encoding them, may be utilized in the various aspects of the invention. Functional variants of toxins and toxin subunits retain the same activity exhibited *in vitro* and/or *in vivo* by the native (non-variant) toxin or subunit (as a toxin or toxin constituent), though the activity may differ in extent or degree. Functional fragments of toxins or toxin subunits retain the same activity exhibited *in vitro* and/or *in vivo* by the full-length toxin or subunit (as a toxin or toxin constituent), though the activity may differ in extent or degree. Functional variants may include one or more sequence alterations anywhere in the polypeptide, *e.g.*, one or both ends and/or in the middle portion. For example, one or more amino acid residues within the sequence can

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be substituted by another amino acid of a similar polarity which acts as a functional variant, resulting in a silent alteration. In some embodiments, conservative substitutions for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs (see Table 2). Conservative substitutions also include

5 substitutions by amino acids having chemically modified side chains that do not eliminate the biological function (as a toxin or toxin constituent) of the resulting variant. It should be understood that although functional fragments and variants retain the activity exhibited by the full-length toxin or subunit or the non-variant toxin or subunit, respectively, truncation or alteration can confer new activities and/or properties and such functional

10 fragments and variants are within the purview of the invention.

Table 2.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

Thus, the invention includes the use of nucleic acids that encode functional

15 variants and functional fragments of specific biological toxins or toxin subunits. A nucleic acid or fragment thereof has substantial identity with another if, when optimally aligned (with appropriate nucleotide insertions or deletions) with the other nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%,

20 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the nucleotide bases. A polypeptide or fragment thereof has substantial identity with another if, when optimally aligned, there is an amino acid sequence identity of at least about 60%, 61%, 62%, 63%,

64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the amino acids.

In some embodiments, the functional variant or functional fragment is a functional
5 variant or functional fragment of a toxin or toxin subunit listed in Table 1. In some
embodiments, the functional variant or functional fragment is a functional variant or
functional fragment of a polypeptide selected from among SEQ ID NO:1, SEQ ID NO:2,
SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID
NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, or SEQ ID
10 NO:13.

The invention includes the use of variant polypeptides comprising amino acid
sequences exhibiting between at least (or at least about) 20.00% to 99.99% (inclusive)
identity to the full length, native, or naturally occurring polypeptide. The aforementioned
range of percent identity is to be taken as including, and providing written description and
15 support for, any fractional percentage, in intervals of 0.01%, between 20.00% and, up to,
including 99.99%. These percentages are purely statistical and differences between two
polypeptide sequences can be distributed randomly and over the entire sequence length.
Thus, variant polypeptides can have 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33,
34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57,
20 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81,
82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identity with
a referenced amino acid sequence or with functional fragments thereof. In one
embodiment, a variant polypeptide exhibits at least 60, 61, 62, 63, 64, 65, 66, 67, 68, 69,
70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93,
25 94, 95, 96, 97, 98, or 99 percent identity to the reference polypeptide. The percent
identity can be calculated with reference to the full-length polypeptide or the length of the
fragment of a particular SEQ ID NO: that is identified.

As indicated above, the invention includes the use of polynucleotides and
polypeptides having substantial identity or substantial similarity (substantial homology)
30 to toxin and toxin subunit sequences disclosed herein. Sequence identity means the
degree of sequence relatedness between two polypeptides or two polynucleotides
sequences as determined by the identity of the match between two strings of such

sequences. Identity can be readily calculated. While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term “identity” is well known to skilled artisans (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). Methods commonly employed to determine identity between two sequences include, but are not limited to those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipman, D., *SIAM J Applied Math.*, 48:1073 (1988). Preferred methods to determine identity are designed to give the largest match between the two sequences tested. Such methods are codified in computer programs. Examples of computer program methods to determine identity between two sequences include, but are not limited to, GCG (Genetics Computer Group, Madison Wis.) program package (Devereux, J., *et al.*, *Nucleic Acids Research*, 1984, 12(l).387), BLASTP, BLASTN, and FASTA. The well-known Smith Waterman algorithm may also be used to determine identity.

Alternatively, substantial homology or similarity exists when a nucleic acid or fragment thereof will hybridize to another nucleic acid (or a complementary strand thereof) under selective hybridization conditions, to a strand, or to its complement. Selectivity of hybridization exists when hybridization which is substantially more selective than total lack of specificity occurs. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent temperature conditions will generally include temperatures in excess of 30 degrees C., typically in excess of 37 degrees C., and preferably in excess of 45 degrees C. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is more important than the measure of any single parameter.

Thus, as herein used, the term “stringent conditions” means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. Such hybridization techniques are well known to those of skill in the art. Stringent hybridization conditions are as defined above or, alternatively, conditions under
5 overnight incubation at 42 degrees C. in a solution comprising: 50% formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5 X Denhardt’s solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1 X SSC at about 65 degrees C.

Methods of purifying a polypeptide expressed in bacteria are well known to a
10 person of ordinary skill in the art and these methods are within the purview of the current invention for purification of the biological toxin or one or more subunits thereof (*e.g.*, extraction, precipitation and differential solubilization, ultracentrifugation, chromatography (size exclusion, charge, hydrophobicity, ion exchange, affinity (*e.g.*, metal binding, immunoaffinity, purification of tagged protein), high performance liquid
15 chromatography). Purification of the biological toxin or subunit(s) may also include one or more concentration steps (*e.g.*, lyophilization, ultrafiltration, denaturing-condition electrophoresis, non-denaturing-condition electrophoresis). Similarly, the methods of assembling different subunits of a protein comprising multiple subunits are also well known to a person of ordinary skill in the art and are within the purview of this invention.

20 Optionally, the biological toxins of the invention will be labeled, so as to be directly detectable, or will be used in conjunction with secondary labeled immunoreagents which will specifically bind the toxin and/or target of the toxin. Labeled toxins may be detected and tracked *in vitro* or *in vivo*. In some embodiments, the label will have a light detectable characteristic. Potential labels include fluorophores, such as
25 fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin and allophycocyanin. Other labels of interest may include dyes, enzymes, chemiluminescers, particles, radioisotopes, or other directly or indirectly detectable agent. The label may be attached to a toxin subunit, or non-toxin subunit, or other constituent of the biological toxin.

30 In an embodiment of the invention, assembling the first subunit and the carrier to produce the hybrid toxin is performed in a container by providing appropriate conditions to facilitate assembly of the biological toxin. The appropriate conditions maintained for

assembling the first subunit and the carrier comprise appropriate temperature, pH, salt concentration, redox environment, and, optionally, protein folding agent. Chemical manipulations to assemble different protein subunits via a plethora of heterobifunctional crosslinking agents are well-known to a person of ordinary skill in the art and such
5 manipulations are within the purview of this invention. For example, commercially available kits can be used to assemble different protein subunits into hybrid toxins.

An ideal preparation of a biological toxin or subunit thereof should be stable, should have low immunogenicity, should have a specific function in determined cells, should have adequate potency with controllable effects, should have long-lasting benefits,
10 and should exert minimal or no side effects. For example, in case of botulinum toxin, the ability to design biological toxin recombinant mixtures may also enhance heavy chain (Hc) terminal binding to the presynaptic protein array, aiding in the control of specific vesicular and trans-membrane “SNAP (Soluble NSF Attachment Protein) Receptor” (SNARE proteins). The clinical uses for biological toxins, for example, BoNT, are still
15 limited; however, clinical interest in the potential role of the biological toxins, for example, neurotoxins, in multiple disorders is increasing. For example, BoNT is likely to be useful for treatment of disorders beyond movement disorders, pain, and other current indications. The understanding of the pathophysiologic mechanisms by which biological toxins, for example, neurotoxins, affect specific target cells, for example, neuronal cells,
20 and the differential effects seen with different serotypes, will aid in tailoring diagnoses and treatments individualized for a patient. To this effect, the current invention provides biological toxins and/or hybrid toxins for such individualized diagnosis and treatment.

The current invention also provides a method of producing a hybrid toxin using a bacterium. The hybrid toxin of the current invention can comprise of a first subunit of a
25 first biological toxin and a carrier, such as a second subunit of a second biological toxin. According to the current invention, the method of producing the hybrid toxin comprises:

- a) introducing in to the bacterium an expressible nucleic acid encoding the first subunit of the first biological toxin and the carrier (for example, a second subunit of a second biological toxin); and
- 30 b) culturing the bacterium under conditions that allow the expression of the first subunit of the first biological toxin and the carrier.

Optionally, the method further comprises c) purifying first subunit of the first biological toxin and the carrier, and, optionally d) assembling first subunit of the first biological toxin and the carrier into the hybrid toxin.

In an embodiment, the hybrid toxin produced by the method of the current invention comprises the first subunit of the first biological toxin which is the active subunit of the first biological toxin and the carrier which is a targeting subunit of a second biological toxin. In another embodiment, the hybrid toxin produced by the method of the current invention comprises the active subunit of botulinum neurotoxin and the targeting subunit of Tetanus toxin. In an even further embodiment, the hybrid toxin produced by the method of the current invention comprises the active subunit of Tetanus toxin and the targeting subunit of botulinum toxin. In an embodiment of the invention, expressing the first subunit and the carrier in a bacterium produces the hybrid toxin in the bacterium without the need of assembling the two or more components (first subunit and carrier). In another embodiment, the first subunit of the first biological toxin and the carrier are purified from the culture of the bacterium and assembled into the hybrid toxin.

Furthermore, the current invention provides a method of producing a hybrid toxin, wherein the hybrid toxin comprises of a first subunit of a first biological toxin and a carrier, such as a second subunit of a second biological toxin, the method comprising:

- a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit of the first biological toxin;
- b) introducing into a second non-pathogenic bacterium an expressible nucleic acid encoding the carrier; and
- c) culturing the first and the second bacteria in one or more culture vessels under conditions that allow for the expression of the first subunit of the first biological toxin and the carrier.

Optionally, the method further comprises d) purifying the first subunit of the first biological toxin and the carrier from the one or more bioreactors; and e) assembling the first subunit of the first biological toxin and the carrier to produce the hybrid toxin.

In an embodiment of the invention, the first bacterium and the second bacterium can be cultured in the same culture vessel, *i.e.* co-cultured. Alternately, the first and the second bacteria can be cultured in separate culture vessels, for example, the first

bacterium is cultured in a first bioreactor and the second bacterium is cultured in a second bioreactor.

In another embodiment of the invention, the first bacterium and the second bacterium belong to the same species and strain. In an embodiment of the invention, the
5 bacterium used to produce a multi-subunit biological toxin is *Lactococcus lactis*.

Alternately, the first bacterium and the second bacterium can belong to different species and strain. When the first bacterium and the second bacterium belong to different species and strain, the first bacterium can belong to the genus *Lactococcus* and the second bacterium can belong to the genus *Bacillus*. In an embodiment of the
10 invention, the first bacterium is *Lactococcus lactis* and the second bacterium is *Bacillus subtilis*.

In an embodiment of the invention, assembling the first subunit and the carrier to produce the hybrid toxin is performed in a container by providing appropriate conditions to facilitate assembly of the hybrid toxin. The appropriate conditions maintained for
15 assembling the first subunit and the carrier comprise appropriate temperature, pH, salt concentration, redox environment, and, optionally, protein folding agent. Chemical manipulations to assemble different protein subunits via a plethora of heterobifunctional crosslinking agents are well-known to a person of ordinary skill in the art and such manipulations are within the purview of this invention. For example, *ad hoc* kits that are
20 commercially available from different sources can be used to assemble different protein subunits into hybrid toxins.

The current invention also provides a hybrid toxin comprising a first subunit of a first biological toxin and a carrier, such as a second subunit of a second biological toxin. In an embodiment, the hybrid toxin of the current invention comprises the active subunit
25 of the first biological toxin and a carrier that is the targeting subunit of a second biological toxin. In another embodiment, the hybrid toxin comprises the active subunit of botulinum neurotoxin and the targeting subunit of Tetanus toxin. In a further embodiment, the hybrid toxin comprises the active subunit of Tetanus toxin and the targeting subunit of botulinum toxin.

30 The current invention provides specifically tailored toxins for individualized treatment. The novel approach to provided biological toxins as provided by the current invention further expands the applications of biological toxins by providing a quick and

relatively safe way to “swap” the properties of different components of biological toxins, as well as to introduce novel ligand moieties that will expand and refine targeting options and ultimately expand the many potential medical applications.

Other aspects of the invention provide hybrid proteins and compositions
5 containing one or more of the hybrid proteins. In these aspects of the invention, a hybrid protein comprises a heterologous sequence fused to the naturally occurring protein. The additional amino acid domain or domains may be located upstream (N-terminal) or downstream (C-terminal) from the sequence of the natural protein. The heterologous sequence may further provide increased stability, targeting or bioavailability of the hybrid
10 protein; or facilitate purification or production of compositions comprising the hybrid proteins. Non-limiting examples of additional amino acid sequences useful for purification or stabilization of the hybrid proteins of the current invention include a maltose binding protein (MBP) sequence, glutathione-S-transferase (GST) sequence, a His tag sequence, a multimerization domain, or the constant region of an immunoglobulin
15 (Ig) molecule.

In certain aspects of the invention, various subdomains of different bacterial toxins are operably linked to a promoter. The term “operably linked” indicates that the polypeptide and additional amino acid domain are associated through peptide linkage, either directly or indirectly, *e.g.*, via spacer residues. In this manner, the hybrid toxins of
20 the current invention can be produced recombinantly, *e.g.*, by direct expression in a host cell. Also, if needed, the additional amino acid sequence included in the hybrid proteins may be eliminated, either at the end of the production/purification process or *in vivo*, *e.g.*, by means of an appropriate endo-/exopeptidase. For example, a spacer sequence included in the hybrid toxins may comprise a recognition site for an endopeptidase (such as a
25 caspase) that can be used to separate by enzymatic cleavage the desired polypeptide from the additional amino acid domain, either *in vivo* or *in vitro*.

In a particular embodiment, the additional amino acid residues in the hybrid toxins comprise the constant region of an immunoglobulin, such as the Fc portion of a human immunoglobulin. The sequence of the Fc portion may be derived, for instance, from an
30 IgG, preferably from a human IgG. The Ig sequence may also be modified to reduce effector function or to increase the stability of a resulting dimer. The amino acid

sequence derived from the constant region of an immunoglobulin may be linked to the C-terminus or to the N-terminus of the naturally occurring full-length protein or subunit.

In a further embodiment, the additional amino acid residues in the hybrid toxins comprise a multimerization domain, allowing complexes to be formed between two or more hybrid toxins of this invention, or between one or more hybrid toxins of this invention and a distinct protein. An example of such multimerization domain includes a leucine zipper. The multimerization domain may be linked to the C-terminus or to the N-terminus of the naturally occurring protein, or both.

An aspect of the invention also provides a polynucleotide that encodes a hybrid toxin of the current invention. The hybrid polynucleotide may include various modifications relative to naturally occurring nucleic acids. The modifications that can be made to naturally occurring nucleic acids include alteration in the naturally occurring nucleotide sequence (*e.g.* substitutions, insertions, deletions, or a combination thereof of one or more nucleotides), fusion with a heterologous nucleotide sequence, attachment of labels, chemical modification of the nucleotides, incorporation of nucleotide analogues, or a combination of two or more of the foregoing modifications.

Further aspects of the invention provide compositions comprising a hybrid toxin of the current invention and one or more pharmaceutically acceptable constituents. The pharmaceutically acceptable constituents include, but are not limited to, excipients, carriers, diluents, fillers, permeation enhancers, solubilizers, chelators, and adjuvants. In certain embodiments, the pharmaceutically acceptable carrier is a non-naturally occurring compound. Examples of pharmaceutically acceptable carriers are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention. In another embodiment, the pharmaceutical composition of the current invention is sterile.

The modular expression of biological toxins in bacteria paves the way to a wide variety of applications. For example, the modular expression of the current invention can be used to deliver active peptides or drugs into specific target cells or tissues, including but not limited to, nervous system, brain, heart, muscle, bone, liver, kidney, pancreas, stomach, intestines, skin, lungs, thymus, adrenal gland, spleen, prostate, testis, ovaries, pituitary gland, thyroid gland, parathyroid gland, pineal gland, other endocrine glands, other exocrine glands, blood, lymphatic tissue, and eyes.

An aspect of the current invention provides a method of targeting a hybrid toxin to a target cell by producing a composition comprising an active subunit (payload) and a targeting subunit (carrier) in a non-pathogenic bacterial cell. For example, the method of producing the hybrid toxins of the current invention can be modified to express different combinations of catalytic light chains and targeting heavy chains of binary biological toxins and multisubunit biological toxins. For example, a catalytic Lc (payload) of a first biological toxin can be directed to a target tissue using a targeting Hc (carrier) from a second biological toxin thereby targeting the Lc of the first biological toxin to a target cell which is not a natural target cell for the first biological toxin. For example, botulinum Lc can be targeted by its own carrier, *i.e.*, botulinum Hc. Alternately, Tetanus Lc can be targeted to a specific target tissue by botulinum Hc. This aspect of the invention can also be manipulated to target therapeutic moieties other than biological toxins, for example, nerve growth factors, whose therapeutic applications are limited by the lack of sites-specific delivery system. An example of this aspect of the invention is described in Fig. 2.

In an embodiment of the invention the payload/carrier combination is designed to target the payload to a target cell which is not a natural target cell for the payload by combining the payload with the carrier that can deliver the payload to the target cell. For example, Tetanus Lc can be combined with botulinum Hc to manufacture a combination useful for delivering Tetanus Lc to a target tissue specific for botulinum Hc.

The carriers used according to current invention include, but are not limited to, BoNT, tetanus toxin binding domains, Adenovirus knob protein from different variants, protein sigma 1 attachment protein from reovirus type 1, shiga-like toxin binding moiety, and rabies attachment protein.

The current invention further provides a method of delivering a biological toxin to a subject, comprising administering to the subject a toxin of the invention (*e.g.*, a hybrid toxin) or a toxin produced according to a method of the invention. In some embodiments, the biological toxin is administered for treatment of a disorder. Thus, another aspect of the invention is a method of treating a disorder in a subject. Treatment of a disorder is intended broadly to include treatment for cosmetic purposes (*e.g.*, as a temporary remedy for wrinkles). In some embodiments, the method of treating a subject according to the invention comprises administering to the subject a pharmaceutically effective amount of a

biological toxin, such as botulinum toxin, wherein the toxin is produced using a bacterium other than *Clostridium botulinum*. In another aspect, the method of treating a subject according to current invention comprises administering to the subject a pharmaceutically effective amount of the hybrid toxin of the current invention. In an embodiment, the method of treating a disorder in a subject comprises administering a pharmaceutically effective amount of a hybrid toxin comprising a first subunit of a first biological toxin and a carrier, such as a second subunit of a second biological toxin, wherein the first subunit of the first biological toxin and the carrier are specifically selected to produce the hybrid toxin which treats the disease in the subject.

Examples of disorders that may be treated with an appropriate biological toxin, such as botulinum toxin, include, but are not limited to, movement disorders (*e.g.*, Parkinson's disease, spasms, and distonias, such as idiopathic focal dystonias, tardive dystonia, and hemifacial spasm/post-facial nerve palsy synkinesis), upper motor neuron syndrome, pain, fibromyalgia, ophthalmological disorders (such as concomitant and nonconcomitant misalignment), involuntary eyelid twitching, involuntary muscle contractions, buttock deformity, anal fissure, hyperhidrosis (excessive sweating), cervical dystonia, migraine and other headache disorders, and achalasia (see, for example, [15] – [20], which are incorporated herein by reference in their entirety).

Biological toxins may be administered to a human or non-human animal subject by any route suitable to effect the intended treatment (for example, applied topically, systemically (*e.g.*, intravenously) or by direct injection into or on a target anatomical site). For example, the biological toxin may be administered intramuscularly, transcutaneously or subcutaneously, intradermally, transconjunctivally, or directly into glands. Delayed or controlled release delivery systems may be used (see, for example [21], which is incorporated herein by reference in its entirety).

Compositions containing the biological toxins of the invention, optionally with one or more diluents and/or other active or non-active constituents, may be formulated for their intended route of administration. Preferably, compositions are suitable for topical application to the skin or mucosa, or for injection. In some embodiments, the composition is a liquid. In some embodiments, the composition is a pharmaceutically acceptable cream, salve, ointment, or gel. Compositions of the invention can be hydrophilic or hydrophobic. The composition can be an aqueous composition, although

other suitable solvents, such as alcohols or other organic solvents, may be used. A combination of solvents may be used. Topically administered compositions may also comprise a matrix in which a biological toxin of the invention is dispersed so that the toxin is held in contact with the skin in order to administer the toxin transdermally.

5 The current invention further provides a bacterium containing an expressible genetic material encoding a heterologous biological toxin (heterologous to the bacterium). In an embodiment of the invention, the bacterium is a non-pathogenic bacterium. In an embodiment of the invention, the bacterium is a bacterium other than *Clostridium spp.* In another embodiment of the invention, the bacterium contains an expressible nucleic acid
10 encoding botulinum neurotoxin. The bacterium can be a gram-positive bacterium or a gram-negative bacterium. The bacterium can be a lactic acid bacterium, for example, lactic acid bacterium belonging to a genus selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus* (e.g., *S. lutetiensis*), *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*,
15 and *Weisella*. If the lactic acid bacterium belongs to the genus *Lactococcus*, it can be selected from the group consisting of *L. lactis*, *L. lactis subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae*, *L. lactis subsp. tructae*, *L. garvieae*, *L. plantarum*, *L. raffinolactis*, *L. piscium*, *L. chungangensis*, and *L. fujiensis*. In an embodiment, the lactic acid bacterium of the current invention is *Lactococcus lactis* subs. *lactis* IL1403.

20 The non-pathogenic bacterium of the current invention can belong to the genus *Bacillus*, for example, non-pathogenic bacterium selected from the group consisting of *B. alcalophilus*, *B. alvei*, *B. aminovorans*, *B. amyloliquefaciens*, *B. aneurinolyticus*, *B. aquaemaris*, *B. atrophaeus*, *B. boroniphilus*, *B. brevis*, *B. caldolyticus*, *B. centrosporus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flavothermus*, *B. fusiformis*, *B. globigii*, *B. infernus*, *B. larvae*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mucilaginosus*, *B. mycoides*, *B. natto*, *B. pantothenicus*, *B. polymyxa*, *B. pseudoanthracis*, *B. pumilus*, *B. schlegelii*, *B. sphaericus*, *B. sporothermodurans*, *B. subtilis*, *B. thermoglucosidasius*, *B. vulgatis*, and *B. weihenstephanensis*. In an
25 embodiment of the current invention, *B. subtilis* is used for the production of the
30 biological toxin according to the method of the current invention.

In a further aspect of the invention, the bacterium used in the method overproduces one or more stabilizing agents. The stabilizing agents that can be used for

that purpose include, but are not limited to, trehalose. In an embodiment, the bacterium used in the method of the current invention is genetically engineered to overproduce stabilizing agents. For example, trehalose biosynthetic operon from *Escherichia coli* can be cloned and introduced into a bacterium which improves the bacterium's tolerance to desiccation. Such bacterium having improved tolerance to desiccation can be used in the method of the current invention.

In a further aspect of the invention, the sequence of the nucleic acid encoding the biological toxin or subunit is optimized for expression in a bacterium of interest. In an embodiment, the sequence of the nucleic acid encoding the biological toxin or subunit is optimized for expression in a gram-negative bacterium or a gram-positive bacterium. Methods of optimizing the sequence of nucleic acid for expression in a gram-positive or a gram-negative bacterium are well known to a person of ordinary skill in the art of molecular biology and are within the purview of this invention. Examples of tools and methods for codon optimization that may be utilized include, but are not limited to, Fulgsang A., "Codon Optimizer: a freeware tool for codon optimization," *Protein Expression and Purification*, 2003, 21:247-249; Puigbo P. *et al.*, "OPTIMIZER: a web server for optimizing the codon usage of DNA sequences," *Nucleic Acids Research*, Web Server Issue, 2007, 35:W126-131; Burgess-Brown N.A. *et al.*, "Codon optimization can improve expression of human genes in *Escherichia coli*: A multi-gene study," *Protein Expression and Purification*, 2008, 59:94-102; and U.S. Patent No. 5,082,767 (Hatfield G.W. *et al.*, "Codon Pair Utilization"), which are each incorporated herein by reference in their entirety.

In a further aspect of the invention, the expression of the biological toxin or subunit in a bacterium is under the control of a promoter. The promoter can be a constitutive promoter or an inducible promoter. In an embodiment of the invention, the promoter is constitutively active in a gram-positive or a gram-negative bacterium. In another embodiment of the invention, the promoter is constitutively active in a gram-positive bacterium. In a further embodiment of the invention, the constitutive promoter is a composite sequence derived from Gram-positive promoters, for example, P24 coupled to p170, the latter of which becomes activated under acidic conditions typically at the end of the growth period for LAB, thus extending the time for protein production. In an

embodiment of the invention, the inducible promoter used to control nucleic acid expression (polypeptide production) is a nisin promoter.

In a further aspect of the invention, the nucleic acid encoding the biological toxin or subunit is designed to contain an mRNA stabilization sequence. The mRNA stabilization sequence can be present as an untranslated mRNA sequence. The untranslated mRNA stabilization sequence can be present at the 5' or the 3' region of the mRNA. Specific sequences and locations of the sequences that stabilize mRNA are well known to a person of ordinary skill in the art and are within the purview of the current invention.

The current invention also provides a method of treating a disorder in a subject, wherein the method comprises administering to the subject a pharmaceutically effective amount of the hybrid toxin of the current invention. An aspect of the current invention provides a method of treating a disorder in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the hybrid toxin which is specifically designed to treat the disorder in the subject.

Exemplified Embodiments

Embodiment 1. A method of producing a biological toxin, or a subunit thereof, using a bacterium other than *Clostridium botulinum*, the method comprising:

a) introducing into the bacterium an expressible nucleic acid encoding the biological toxin or subunit; and

b) culturing the bacterium containing the introduced nucleic acid under conditions that allow expression of the nucleic acid and production of the biological toxin or subunit.

Embodiment 2. The method of embodiment 1, further comprising: c) purifying the biological toxin or subunit from the culture of the bacterium.

Embodiment 3. The method of embodiment 1, wherein the biological toxin comprises multiple subunits.

Embodiment 4. The method of embodiment 1, wherein the biological toxin is botulinum neurotoxin.

Embodiment 5. The method of embodiment 1, wherein the bacterium is a non-pathogenic bacterium.

Embodiment 6. The method of embodiment 1, wherein the bacterium is a gram-positive bacterium or a gram-negative bacterium.

Embodiment 7. The method of embodiment 1, wherein the bacterium is a lactic acid bacterium.

5 Embodiment 8. The method of embodiment 7, wherein the lactic acid bacterium belongs to the genus selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*.

10 Embodiment 9. The method of embodiment 7, wherein the lactic acid bacterium is a member of the genus *Lactococcus*.

Embodiment 10. The method of embodiment 8, wherein the lactic acid bacterium is selected from the group consisting of *L. lactis*, *L. lactis subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae*, *L. lactis subsp. tructae*, *L. garvieae*, *L. plantarum*, *L. raffinolactis*, *L. piscium*, *L. chungangensis*, and *L. fujiensis*.

15 Embodiment 11. The method of embodiment 10, wherein the lactic acid bacterium is *Lactococcus lactis*.

Embodiment 12. The method of embodiment 1, wherein the bacterium belongs to the genus *Bacillus*.

20 Embodiment 13. The method of embodiment 12, wherein the bacterium belongs to a species selected from the group consisting of *B. alcalophilus*, *B. alvei*, *B. aminovorans*, *B. amyloliquefaciens*, *B. aneurinoliticus*, *B. aquaemaris*, *B. atrophaeus*, *B. boroniphilus*, *B. brevis*, *B. caldolyticus*, *B. centrosporus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flavothermus*, *B. fusiformis*, *B. globigii*, *B. infernus*, *B. larvae*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mucilaginosus*, *B. mycoides*, *B. natto*, *B. pantothenicus*, *B. polymyxa*, *B. pseudoanthracis*, *B. pumilus*, *B. schlegelii*, *B. sphaericus*, *B. sporothermodurans*, *B. subtilis*, *B. thermoglucosidasius*, *B. vulgatis*, and *B. weihenstephanensis*.

Embodiment 14. The method of embodiment 13, wherein the bacterium is *Bacillus subtilis*.

30 Embodiment 15. A method of producing a biological toxin comprising a first subunit and a second subunit, the method comprising:

a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit;

b) introducing into a second bacterium an expressible nucleic acid encoding the second subunit; and

5 c) culturing the first and the second bacteria containing the expressible nucleic acids under conditions that allow expression of the first subunit and the second subunit.

Embodiment 16. The method of embodiment 15, further comprising: d) purifying the first subunit and the second subunit from the culture of the first bacterium and the second bacterium.

10 Embodiment 17. The method of embodiment 16, further comprising: e) assembling the first subunit and the second subunit to produce the biological toxin.

Embodiment 18. The method of embodiment 15, wherein the first bacterium and/or the second bacterium are non-pathogenic bacterium.

15 Embodiment 19. The method of embodiment 15, wherein both the first bacterium and the second bacterium are cultured in the same culture vessel.

Embodiment 20. The method of embodiment 15, wherein the first bacterium and the second bacterium are cultured in separate culture vessels.

Embodiment 21. The method of embodiment 15, wherein the first bacterium and the second bacterium belong to the same species and strain.

20 Embodiment 22. The method of embodiment 15, wherein both the first bacterium and the second bacterium are lactic acid bacteria.

Embodiment 23. The method of embodiment 15, wherein both the first bacterium and the second bacterium are *Lactococcus lactis*.

25 Embodiment 24. The method of embodiment 15, wherein the first bacterium and the second bacterium belong to different species and strain.

Embodiment 25. The method of embodiment 24, wherein the first bacterium belong to the genus *Lactococcus* and the second bacterium belong to the genus *Bacillus*.

30 Embodiment 26. The method of embodiment 17, wherein assembling the first subunit of the first biological toxin and the second subunit of the second biological toxin to produce the biological toxin is performed in a container by providing appropriate conditions.

Embodiment 27. The method of embodiment 26, wherein the appropriate conditions comprise appropriate temperature, pH, salt concentration, redox environment, and protein folding agent.

Embodiment 28. A bacterium containing an expressible nucleic acid
5 encoding a heterologous biological toxin or subunit thereof.

Embodiment 29. The bacterium of embodiment 28, wherein the bacterium is non-pathogenic.

Embodiment 30. The bacterium of embodiment 28, wherein the biological toxin is botulinum neurotoxin.

10 Embodiment 31. The bacterium of embodiment 28, wherein the bacterium is other than *Clostridium spp.*

Embodiment 32. The bacterium of embodiment 28, wherein the bacterium is a gram-positive bacterium or a gram-negative bacterium.

15 Embodiment 33. The bacterium of embodiment 28, wherein the bacterium is a lactic acid bacterium.

Embodiment 34. The bacterium of embodiment 33, wherein the lactic acid bacterium belongs to a genus selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*.

20 Embodiment 35. The bacterium of embodiment 34, wherein the lactic acid bacterium belongs to the genus *Lactococcus*.

Embodiment 36. The bacterium of embodiment 35, wherein the lactic acid bacterium is selected from the group consisting of *L. lactis*, *L. lactis subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae*, *L. lactis subsp. tructae*, *L. garvieae*, *L. plantarum*, *L. raffinolactis*, *L. piscium*, *L. chungangensis*, and *L. fujiensis*.
25

Embodiment 37. The bacterium of embodiment 36, wherein the lactic acid bacterium is *Lactococcus lactis*.

Embodiment 38. The bacterium of embodiment 36, wherein the lactic acid bacterium is *Lactococcus lactis* IL1403.

30 Embodiment 39. The bacterium of embodiment 28, wherein the bacterium belongs to the genus *Bacillus*.

Embodiment 40. The bacterium of embodiment 39, wherein the bacterium is selected from the group consisting of *B. alcalophilus*, *B. alvei*, *B. aminovorans*, *B. amyloliquefaciens*, *B. aneurinolyticus*, *B. aquaemaris*, *B. atrophaeus*, *B. boroniphilus*, *B. brevis*, *B. caldolyticus*, *B. centrosporus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flavothermus*, *B. fusiformis*, *B. globigii*, *B. infernus*, *B. larvae*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mucilaginosus*, *B. mycoides*, *B. natto*, *B. pantothenicus*, *B. polymyxa*, *B. pseudoanthracis*, *B. pumilus*, *B. schlegelii*, *B. sphaericus*, *B. sporothermodurans*, *B. subtilis*, *B. thermoglucosidasius*, *B. vulgatis*, and *B. weihenstephanensis*.

10 Embodiment 41. The bacterium of embodiment 40, wherein the bacterium is *Bacillus subtilis*.

Embodiment 42. A method of producing a hybrid toxin using a bacterium, wherein the hybrid toxin comprises of a first subunit of a first biological toxin and a carrier, the method comprising:

15 a) introducing into the bacterium a expressible nucleic acid encoding the first subunit of the first biological toxin and the carrier; and

 b) culturing the bacterium in conditions that allow for the expression of the first subunit of the first biological toxin and the carrier.

20 Embodiment 43. The method of embodiment 42, further comprising c) purifying the hybrid toxin.

Embodiment 44. The method of embodiment 42, wherein the bacterium is non-pathogenic.

Embodiment 45. The method of embodiment 42, wherein the bacterium belongs to the genus *Lactococcus*.

25 Embodiment 46. The method of embodiment 42, wherein the bacterium is *Lactococcus lactis*.

Embodiment 47. The method of embodiment 42, wherein the carrier is a subunit of a second biological toxin.

30 Embodiment 48. The method of embodiment 42, wherein the first subunit of the first biological toxin is the active subunit of the first biological toxin and the carrier is a targeting subunit of a second biological toxin.

Embodiment 49. The method of embodiment 42, wherein the first subunit of the first biological toxin is the active subunit of botulinum neurotoxin and the carrier is selected from among a targeting subunit of Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

5 Embodiment 50. The method of embodiment 42, wherein the first subunit of the first biological toxin is the active subunit of Tetanus toxin and the carrier is the targeting subunit of botulinum toxin.

Embodiment 51. A method of producing a hybrid toxin, wherein the hybrid toxin comprises of a first subunit of a first biological toxin and a carrier, the method
10 comprising:

 a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit of the first biological toxin;

 b) introducing into a second bacterium an expressible nucleic acid encoding the carrier; and

15 c) culturing the first and the second bacteria in one or more bioreactors under conditions that allow for the expression of the first subunit of the first biological toxin and the carrier.

Embodiment 52. The method of embodiment 51, further comprising d) purifying the first subunit of the first biological toxin and the carrier from the one or more
20 bioreactors.

Embodiment 53. The method of embodiment 52, further comprising e) assembling the first subunit of the first biological toxin and the carrier to manufacture the hybrid toxin.

Embodiment 54. The method of embodiment 51, wherein the first bacterium
25 and/or the second bacterium is non-pathogenic.

Embodiment 55. The method of embodiment 51, wherein the first bacterium and the second bacterium are cultured in the same culture vessel.

Embodiment 56. The method of embodiment 51, wherein the first bacterium and the second bacterium are cultured in different bioreactors.

30 Embodiment 57. The method of embodiment 51, wherein the first and the second bacteria belong to the same species and strain.

Embodiment 58. The method of embodiment 51, wherein both the first bacterium and the second bacterium are lactic acid bacteria.

Embodiment 59. The method of embodiment 51, wherein both the first bacterium and the second bacterium are *Lactococcus lactis*.

5 Embodiment 60. The method of embodiment 51, wherein the first bacterium and the second bacterium belong to different species or strain.

Embodiment 61. The method of embodiment 51, wherein the first bacterium belongs to the genus *Lactococcus* and the second bacterium belongs to the genus *Bacillus*.

10 Embodiment 62. The method of embodiment 52, wherein assembling the first subunit of the first biological toxin and the carrier to manufacture the hybrid toxin is performed in a container by providing appropriate conditions.

Embodiment 63. The method of embodiment 62, wherein the appropriate conditions comprise appropriate temperature, pH, salt concentration, redox environment, and, optionally, protein folding agent.

15 Embodiment 64. The method of embodiment 51, wherein the carrier is a subunit of a second biological toxin.

Embodiment 65. The method of embodiment 51, wherein the carrier is selected from among a targeting subunit of Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

20 Embodiment 66. A hybrid toxin comprising a first subunit of a first biological toxin and a carrier.

Embodiment 67. The hybrid toxin of embodiment 66, wherein the carrier is a subunit of a second biological toxin.

25 Embodiment 68. The hybrid toxin of embodiment 66, wherein the first subunit of the first biological toxin is the active subunit of the first biological toxin and the carrier is the targeting subunit of the second biological toxin.

30 Embodiment 69. The hybrid toxin of embodiment 66, wherein the first subunit of the first biological toxin is the active subunit of botulinum toxin and the carrier is selected from among a targeting subunit of the Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

Embodiment 70. The hybrid toxin of embodiment 66, wherein the first subunit of the first biological toxin is the active subunit of Tetanus toxin and the carrier is the targeting subunit of botulinum toxin

Embodiment 71. A method of delivering a toxin, toxin subunit, or hybrid toxin to a subject, comprising administering to the subject the toxin, toxin subunit, or hybrid toxin of any preceding embodiment.

Embodiment 72. A method of treating a disorder in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the toxin, subunit or hybrid toxin of any preceding embodiment.

Embodiment 73. A method of treating a disorder in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the hybrid toxin of any preceding embodiment, wherein the first subunit of the first biological toxin and the carrier are specifically selected to treat the disorder in the subject.

Embodiment 74. A biological toxin or hybrid toxin produced by a method of any one of embodiments 1-27 or 42-65.

Embodiment 75. A composition comprising a biological toxin or hybrid toxin of any preceding embodiment.

Embodiment 76. The composition of embodiment 75, wherein the composition is a liquid, cream, salve, ointment, or gel.

Further Definitions

As used herein, the term “nucleic acid” refers to genetic material including, but not limited to, RNA, DNA, cDNA, *etc.* Further, expressible nucleic acid is the genetic material capable of being transcribed and translated into proteins. Examples of expressible nucleic acid include, but are not limited to, plasmids containing genes that are transcribed and translated into proteins by the host cell, genes that are incorporated in to the genome of a host cell that are transcribed and translated into proteins, RNA translated into proteins, DNA transcribed and translated into proteins *in vitro*, and RNA translated into proteins *in vitro*. Additional examples of expressible nucleic acid are well known to a person of ordinary skill in the art and are within the purview of the current invention. Expressible nucleic acid can encode one or more polypeptides.

As used herein, the terms “oligopeptide”, “polypeptide”, “peptide” and “protein” are used interchangeably to refer to a series of residues of any length (two or more), typically L-amino acids, connected one to the other, typically by peptide bonds between the α -amino and carboxyl groups of adjacent amino acids. Linker elements can be joined to the polypeptides of the subject invention, for example, through peptide bonds or via chemical bonds (*e.g.*, heterobifunctional chemical linker elements) as set forth below. Additionally, the terms “amino acid(s)” and “residue(s)” can be used interchangeably. Polypeptides according to the instant invention may also contain non-natural amino acids. In some embodiments, the polypeptides are not in their natural environment but may have been isolated or obtained by purification from natural sources or obtained from cultures or host cells prepared by genetic manipulation (*e.g.*, the peptides, or fragments thereof, are recombinantly produced by host cells, or by chemical synthesis).

As used herein, the phrase “introducing a nucleic acid into a bacterium” comprises inserting the nucleic acid into one or more bacterium in such a way that the genetic material can be expressed into polypeptides. The genetic material introduced into the bacterium can exist independently of the bacterial genome, for example, a plasmid, or the genetic material introduced in to the bacterium can get incorporated into the bacterial genome.

For the purposes of this invention, the conditions that allow expression of expressible nucleic acids include, but are not limited to, providing an appropriate host for the expression, appropriate culture medium, temperature, pH, availability of inducers when inducible promoters are used, *etc.* Appropriate culture conditions that allow for the expression of nucleic acids are well known to a person of ordinary skill in the art and are within the purview of this invention.

As used herein, the term “bioreactor” refers to an apparatus for growing microorganisms, such as bacteria or yeast, under controlled conditions, and that are used in the production of a biological substance. Various types of bioreactors are well known to a person of ordinary skill in the art and are within the purview of the current invention. Bioreactors typically include a bioreactor vessel (a container that holds the media and cells) and ports for input and output of material. Bioreactors may also include an agitator, sparger, baffles, probes (temperature, dissolved oxygen, pH, pressure gauge), cooling jacket, condenser, back pressure valve, inlet filters, exhaust filters, valves, and a load cell.

The culture media utilized can be defined or undefined as appropriate for the culture of the bacteria and production of the toxin. Examples of media include, but are not limited to, enriched media and selective media.

As used herein, the terms “treating,” “treatment”, and grammatical variations thereof refer to the cure of the disorder, mitigation of the disorder, alleviation or elimination one or more symptoms of the disorder, delay in the onset of the disorder, or prevention of the disorder or other undesirable condition.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting.

EXAMPLE 1 – PRODUCING BOTULINUM NEUROTOXIN IN *LACTOCOCCUS LACTIS*

The overall modular strategy used for DNA constructs is different from those reported in the literature for *E. coli* and the expression is optimized in Gram-positive bacteria and expressed in *Lactococcus lactis* as opposed to *E. coli*. Understanding that the Lc needs its own cognate heavy chain N-terminus (Hn) to execute effective delivery, other groups have fused Lc to Hn and have expressed heavy chain C-terminus (Hc; the binding moiety) as a separate protein. In contrast, according to an embodiment of the current invention, Lc is expressed as a separate protein and Hn is fused to Hc through a GGGCCC ApaI site in-frame to provide for a Pro-Gly flexible linker.

The LAB expression was designed to operate using two different promoters allowing flexibility for expression of BoNT modules in two different embodiments. One embodiment uses the nisin promoter, which is inducible and leads to a more stable expression. The other promoter is unique and it is constitutive. This constitutive promoter is a composite sequence derived from a broad range of Gram-positive promoters, for example, P24 coupled to p170, which becomes activated under acidic

conditions typically at the end of growth period for LAB. Each component of the promoter also contains untranslated sequences that stabilize the mRNA.

The coding sequences for the genes are optimized for expression in *Lactococcus lactis*, which can also be modified for expression in any *Lactococcus spp.* or any related species. The starting material is pLactoBoNT/A-Lc, provided by Genescript in pUC57 and oriented 5'-3' HindIII-EcoRI. The synthetic DNA coding for catalytically inactive Lc contains a NotI-stuffer-SmaI sequence downstream of the STOP codon to enable further cloning. Using the XbaI site in the vector polylinker sequence, the promoter-Lc cassette as a BglIII-XbaI fragment was cloned into the vector, pMSP3535H3. This cloning resulted in the removal of the resident nisin-inducible promoter and generated pBzMM200 (Fig. 1A). Insertion of Hn and Hc as separate genes downstream of Lc generated pBzMM201 (Fig. 1B) which failed to produce any detectable Hc and was therefore discarded. As an alternative, Hn was provided with NotI and ApaI ends, and Hc with ApaI and SmaI ends. Then, a tripartite ligation was set whereby pBzMM200, restricted with NotI and SmaI was closed by bridging both Hn (NotI-ApaI) and Hc (ApaI-SmaI). The results from this procedure are illustrated in the map for pGnMM1 (Fig. 1C). This version of the modular Hn-Hc (heavy chain) was also expressed separately under the nisin-inducible promoter by adding AgeI sites at both ends and cloning it into the pMSP3535H3 and cutting with AgeI. This procedure generated pGnMM2 (Fig. 1D). The same strategy was used for expressing Hc alone with pGnMM3 (Fig. 1E) and, again, Lc together with Hn-Hc as inducible proteins with pGnMM5 (Fig. 1F).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

REFERENCES

1. Jankovic, J. Disease-oriented approach to botulinum toxin use. *Toxicon* **2009**, *54*, 614–623.
- 5 2. Olesen, J.; Tfelt-Hansen, P. Licence for Botox in so-called chronic migraine. *Lancet* **2010**, *376*, 1825–1826; discussion 1826.
3. FDA approves Botox to treat specific form of urinary incontinence. Available online:
10 www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm269509.htm
(accessed on 24 October 2011).
4. Hasler, W.L. The expanding spectrum of clinical uses for botulinum toxin: Healing of chronic anal fissures. *Gastroenterology* **1999**, *116*, 221–223.
- 15 5. Dykstra, D.D.; Sidi, A.A. Treatment of detrusor-sphincter dyssynergia with botulinum A toxin: A double-blind study. *Arch. Phys. Med. Rehabil.* **1990**, *71*, 24–26.
- 20 6. Snow, B.J.; Tsui, J.K.; Bhatt, M.H.; Varelas, M.; Hashimoto, S.A.; Calne, D.B. Treatment of spasticity with botulinum toxin: a double-blind study. *Ann. Neurol.* **1990**, *28*, 512–515.
- 25 7. Das, T.K.; Park, D.M. Effect of treatment with botulinum toxin on spasticity. *Postgrad. Med. J.* **1989**, *65*, 208–210.
8. Rollnik, J.D.; Tanneberger, O.; Schubert, M.; Schneider, U.; Dengler, R. Treatment of tension-type headache with botulinum toxin type A: A double-blind, placebo-controlled study. *Headache* **2000**, *40*, 300–305.
- 30 9. Carruthers, A.; Carruthers, J. *Botulinum* toxin products overview. *Skin Ther. Lett.* **2008**, *13*, 1–4.
10. EXS. 2010;100:1-29. Toxins from bacteria. Henkel JS, Baldwin MR, Barbieri JT.
- 35 11. Curr. Med Chem. 2008;15(5):459-69. Binary actin-ADP-ribosylating toxins and their use as molecular Trojan horses for drug delivery into eukaryotic cells. Barth H, Stiles BG.
- 40 12. Botulinum neurotoxin: a marvel of protein design. Montal M. Annu Rev Biochem. 2010; 79:591-617. doi: 10.1146/annurev.biochem.051908.125345. Review.
13. <http://www.ncbi.nlm.nih.gov/pubmed/22141396>, FEBS J. 2012; 279(3):515-23. doi: 10.1111/j.1742-4658.2011.08444.x. Stancombe PR, Masuyer G, Birch-Machin I, Beard M, Foster KA, Chaddock JA, Acharya KR.
- 45 14. Expression and purification of the light chain of botulinum neurotoxin A: a single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution

with the heavy chain. <http://www.ncbi.nlm.nih.gov/pubmed/7578132>. 1995; 34(46):15175-81. Zhou L, de Paiva A, Liu D, Aoki R, Dolly JO.

- 5 15. Uses of botulinum toxin injection in medicine today. BMJ. 2000, 320:161-165. Munchau A, Bhatia KP.
16. Botulinum toxin: clinical use. Parkinsonism Relat. Disord. 2006; Sep. 12(6):331-355. Epub 2006 Jul 25. Truong DD, Jost WH.
- 10 17. The use of botulinum toxin in physical medicine and rehabilitation. Bol. Asoc. Med. P R. 2006 Jan-Mar. 98(1):42-55. Cuevas-Tristan RL, Cruz-Jimenez M.
18. The current use of botulinum toxin. J. Clin. Neurosci. 2000; Sep. 7(5):389-394. Mahant N, Clouston PD, Lorentz IT.
- 15 19. Management of spasticity in adults: practical application of botulinum toxin. Eur. J. neurol. 2006; Feb. 13 Suppl 1:42-50. Pathak MS, Nguyen HT, Graham HK, Moore AP.
- 20 20. Botulinum toxin in movement disorders. Semin. Neurol. 2007; Apr. 27(2):183-194. Papapetropoulos S, Singer C.
21. Botulinum toxin implant. U.S. Patent Publication Number 20020028216. Published March 7, 2002. Donovan S.
- 25

CLAIMS

We claim:

1. A method of producing a biological toxin, or a subunit thereof, using a bacterium other than *Clostridium botulinum*, the method comprising:
 - a) introducing into the bacterium an expressible nucleic acid encoding the biological toxin or subunit; and
 - b) culturing the bacterium containing the introduced nucleic acid under conditions that allow expression of the nucleic acid and production of the biological toxin or subunit.
2. The method of claim 1, further comprising: c) purifying the biological toxin or subunit from the culture of the bacterium.
3. The method of claim 1, wherein the biological toxin comprises multiple subunits.
4. The method of claim 1, wherein the biological toxin is botulinum neurotoxin.
5. The method of claim 1, wherein the bacterium is a non-pathogenic bacterium.
6. The method of claim 1, wherein the bacterium is a gram-positive bacterium or a gram-negative bacterium.
7. The method of claim 1, wherein the bacterium is a lactic acid bacterium.
8. The method of claim 7, wherein the lactic acid bacterium belongs to the genus selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*.
9. The method of claim 7, wherein the lactic acid bacterium is a member of the genus *Lactococcus*.

10. The method of claim 8, wherein the lactic acid bacterium is selected from the group consisting of *L. lactis*, *L. lactis subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae*, *L. lactis subsp. tructae*, *L. garvieae*, *L. plantarum*, *L. raffinolactis*, *L. piscium*, *L. chungangensis*, and *L. fujiensis*.

11. The method of claim 10, wherein the lactic acid bacterium is *Lactococcus lactis*.

12. The method of claim 1, wherein the bacterium belongs to the genus *Bacillus*.

13. The method of claim 12, wherein the bacterium belongs to a species selected from the group consisting of *B. alcalophilus*, *B. alvei*, *B. aminovorans*, *B. amyloliquefaciens*, *B. aneurinolyticus*, *B. aquaemaris*, *B. atrophaeus*, *B. boroniphilus*, *B. brevis*, *B. caldolyticus*, *B. centrosporus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flavothermus*, *B. fusiformis*, *B. globigii*, *B. infernus*, *B. larvae*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mucilaginosus*, *B. mycoides*, *B. natto*, *B. pantothenicus*, *B. polymyxa*, *B. pseudoanthracis*, *B. pumilus*, *B. schlegelii*, *B. sphaericus*, *B. sporothermodurans*, *B. subtilis*, *B. thermoglucosidasius*, *B. vulgatis*, and *B. weihenstephanensis*.

14. The method of claim 13, wherein the bacterium is *Bacillus subtilis*.

15. A method of producing a biological toxin comprising a first subunit and a second subunit, the method comprising:

a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit;

b) introducing into a second bacterium an expressible nucleic acid encoding the second subunit; and

c) culturing the first and the second bacteria containing the expressible nucleic acids under conditions that allow expression of the first subunit and the second subunit.

16. The method of claim 15, further comprising: d) purifying the first subunit and the second subunit from the culture of the first bacterium and the second bacterium.

17. The method of claim 16, further comprising: e) assembling the first subunit and the second subunit to produce the biological toxin.

18. The method of claim 15, wherein the first bacterium and/or the second bacterium are non-pathogenic bacterium.

19. The method of claim 15, wherein both the first bacterium and the second bacterium are cultured in the same culture vessel.

20. The method of claim 15, wherein the first bacterium and the second bacterium are cultured in separate culture vessels.

21. The method of claim 15, wherein the first bacterium and the second bacterium belong to the same species and strain.

22. The method of claim 15, wherein both the first bacterium and the second bacterium are lactic acid bacteria.

23. The method of claim 15, wherein both the first bacterium and the second bacterium are *Lactococcus lactis*.

24. The method of claim 15, wherein the first bacterium and the second bacterium belong to different species and strain.

25. The method of claim 24, wherein the first bacterium belong to the genus *Lactococcus* and the second bacterium belong to the genus *Bacillus*.

26. The method of claim 17, wherein assembling the first subunit of the first biological toxin and the second subunit of the second biological toxin to produce the biological toxin is performed in a container by providing appropriate conditions.

27. The method of claim 26, wherein the appropriate conditions comprise appropriate temperature, pH, salt concentration, redox environment, and protein folding agent.

28. A bacterium containing an expressible nucleic acid encoding a heterologous biological toxin or subunit thereof.

29. The bacterium of claim 28, wherein the bacterium is non-pathogenic.

30. The bacterium of claim 28, wherein the biological toxin is botulinum neurotoxin.

31. The bacterium of claim 28, wherein the bacterium is other than *Clostridium spp.*

32. The bacterium of claim 28, wherein the bacterium is a gram-positive bacterium or a gram-negative bacterium.

33. The bacterium of claim 28, wherein the bacterium is a lactic acid bacterium.

34. The bacterium of claim 33, wherein the lactic acid bacterium belongs to a genus selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*.

35. The bacterium of claim 34, wherein the lactic acid bacterium belongs to the genus *Lactococcus*.

36. The bacterium of claim 35, wherein the lactic acid bacterium is selected from the group consisting of *L. lactis*, *L. lactis subsp. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. hordniae*, *L. lactis subsp. tructae*, *L. garvieae*, *L. plantarum*, *L. raffinolactis*, *L. piscium*, *L. chungangensis*, and *L. fujiensis*.

37. The bacterium of claim 36, wherein the lactic acid bacterium is *Lactococcus lactis*.

38. The bacterium of claim 36, wherein the lactic acid bacterium is *Lactococcus lactis* IL1403.

39. The bacterium of claim 28, wherein the bacterium belongs to the genus *Bacillus*.

40. The bacterium of claim 39, wherein the bacterium is selected from the group consisting of *B. alcalophilus*, *B. alvei*, *B. aminovorans*, *B. amyloliquefaciens*, *B. aneurinolyticus*, *B. aquaemaris*, *B. atrophaeus*, *B. boroniphilus*, *B. brevis*, *B. caldolyticus*, *B. centrosporus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flavothermus*, *B. fusiformis*, *B. globigii*, *B. infernus*, *B. larvae*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mucilaginosus*, *B. mycoides*, *B. natto*, *B. pantothenicus*, *B. polymyxa*, *B. pseudoanthracis*, *B. pumilus*, *B. schlegelii*, *B. sphaericus*, *B. sporothermodurans*, *B. subtilis*, *B. thermoglucosidasius*, *B. vulgatis*, and *B. weihenstephanensis*.

41. The bacterium of claim 40, wherein the bacterium is *Bacillus subtilis*.

42. A method of producing a hybrid toxin using a bacterium, wherein the hybrid toxin comprises of a first subunit of a first biological toxin and a carrier, the method comprising:

a) introducing into the bacterium a expressible nucleic acid encoding the first subunit of the first biological toxin and the carrier; and

b) culturing the bacterium in conditions that allow for the expression of the first subunit of the first biological toxin and the carrier.

43. The method of claim 42, further comprising c) purifying the hybrid toxin.

44. The method of claim 42, wherein the bacterium is non-pathogenic.

45. The method of claim 42, wherein the bacterium belongs to the genus *Lactococcus*.

46. The method of claim 42, wherein the bacterium is *Lactococcus lactis*.

47. The method of claim 42, wherein the carrier is a subunit of a second biological toxin.

48. The method of claim 42, wherein the first subunit of the first biological toxin is the active subunit of the first biological toxin and the carrier is a targeting subunit of a second biological toxin.

49. The method of claim 42, wherein the first subunit of the first biological toxin is the active subunit of botulinum neurotoxin and the carrier is selected from among a targeting subunit of Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

50. The method of claim 42, wherein the first subunit of the first biological toxin is the active subunit of Tetanus toxin and the carrier is the targeting subunit of botulinum toxin.

51. A method of producing a hybrid toxin, wherein the hybrid toxin comprises of a first subunit of a first biological toxin and a carrier, the method comprising:

a) introducing into a first bacterium an expressible nucleic acid encoding the first subunit of the first biological toxin;

b) introducing into a second bacterium an expressible nucleic acid encoding the carrier; and

c) culturing the first and the second bacteria in one or more bioreactors under conditions that allow for the expression of the first subunit of the first biological toxin and the carrier.

52. The method of claim 51, further comprising d) purifying the first subunit of the first biological toxin and the carrier from the one or more bioreactors.

53. The method of claim 52, further comprising e) assembling the first subunit of the first biological toxin and the carrier to manufacture the hybrid toxin.

54. The method of claim 51, wherein the first bacterium and/or the second bacterium is non-pathogenic.

55. The method of claim 51, wherein the first bacterium and the second bacterium are cultured in the same culture vessel.

56. The method of claim 51, wherein the first bacterium and the second bacterium are cultured in different bioreactors.

57. The method of claim 51, wherein the first and the second bacteria belong to the same species and strain.

58. The method of claim 51, wherein both the first bacterium and the second bacterium are lactic acid bacteria.

59. The method of claim 51, wherein both the first bacterium and the second bacterium are *Lactococcus lactis*.

60. The method of claim 51, wherein the first bacterium and the second bacterium belong to different species or strain.

61. The method of claim 51, wherein the first bacterium belongs to the genus *Lactococcus* and the second bacterium belongs to the genus *Bacillus*.

62. The method of claim 52, wherein assembling the first subunit of the first biological toxin and the carrier to manufacture the hybrid toxin is performed in a container by providing appropriate conditions.

63. The method of claim 62, wherein the appropriate conditions comprise appropriate temperature, pH, salt concentration, redox environment, and, optionally, protein folding agent.

64. The method of claim 51, wherein the carrier is a subunit of a second biological toxin.

65. The method of claim 51, wherein the carrier is selected from among a targeting subunit of Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

66. A hybrid toxin comprising a first subunit of a first biological toxin and a carrier.

67. The hybrid toxin of claim 66, wherein the carrier is a subunit of a second biological toxin.

68. The hybrid toxin of claim 66, wherein the first subunit of the first biological toxin is the active subunit of the first biological toxin and the carrier is the targeting subunit of the second biological toxin.

69. The hybrid toxin of claim 66, wherein the first subunit of the first biological toxin is the active subunit of botulinum toxin and the carrier is selected from among a targeting subunit of the Tetanus toxin, adenovirus knob, reovirus protein sigma 1, Shiga-like toxin binding moiety, or rabies attachment protein.

70. The hybrid toxin of claim 66, wherein the first subunit of the first biological toxin is the active subunit of Tetanus toxin and the carrier is the targeting subunit of botulinum toxin.

71. A method of delivering a toxin, toxin subunit, or hybrid toxin to a subject, comprising administering to the subject the toxin, toxin subunit, or hybrid toxin of any preceding claim.

72. A method of treating a disorder in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the toxin, subunit or hybrid toxin of any preceding claim.

73. A method of treating a disorder in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the hybrid toxin of any preceding claim, wherein the first subunit of the first biological toxin and the carrier are specifically selected to treat the disorder in the subject.

74. A biological toxin or hybrid toxin produced by a method of any one of claims 1-27 or 42-65.

75. A composition comprising a biological toxin or hybrid toxin of any one of claims 1-27 or 42-70.

76. The composition of claim 75, wherein the composition is a liquid, cream, salve, ointment, or gel.

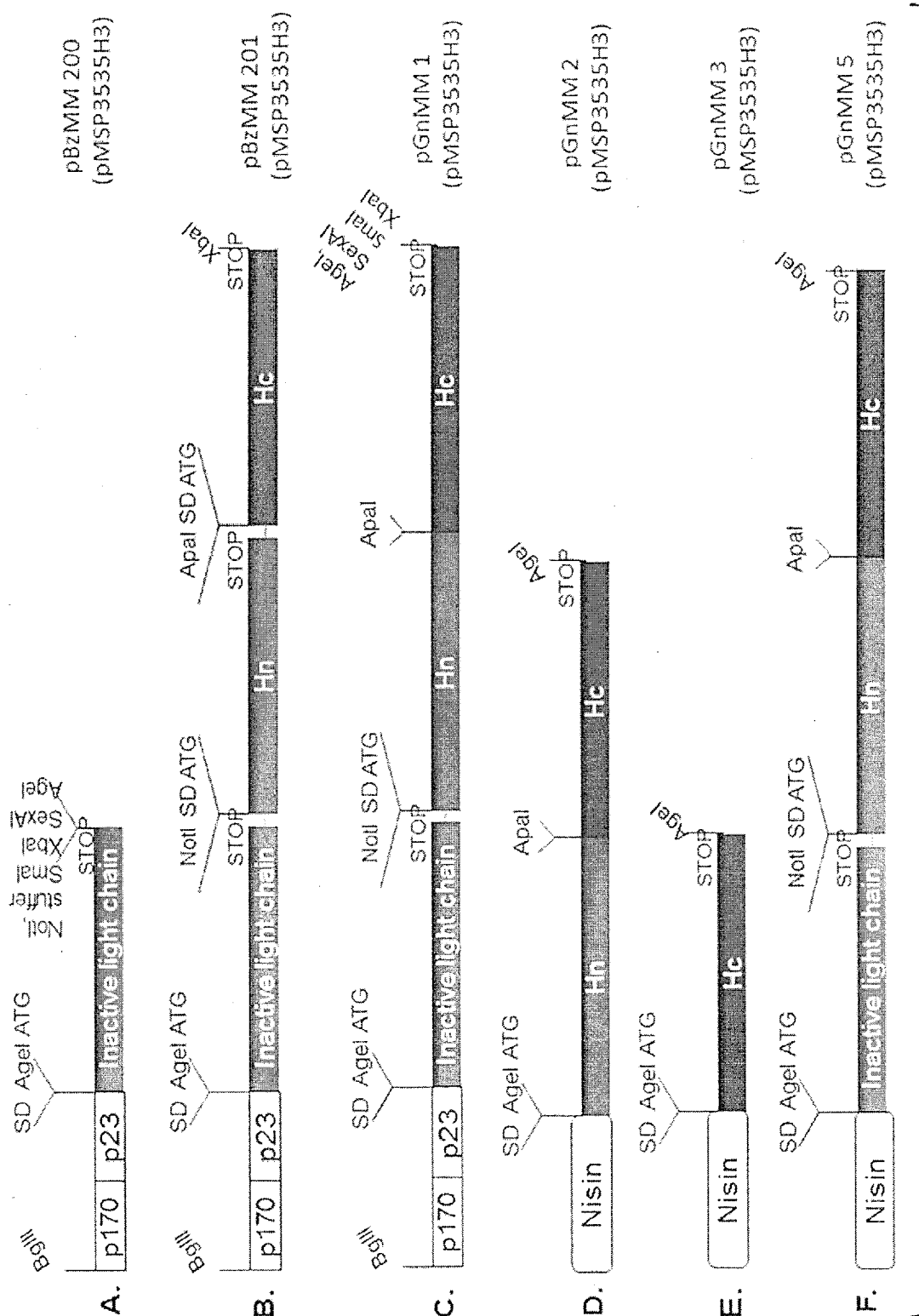
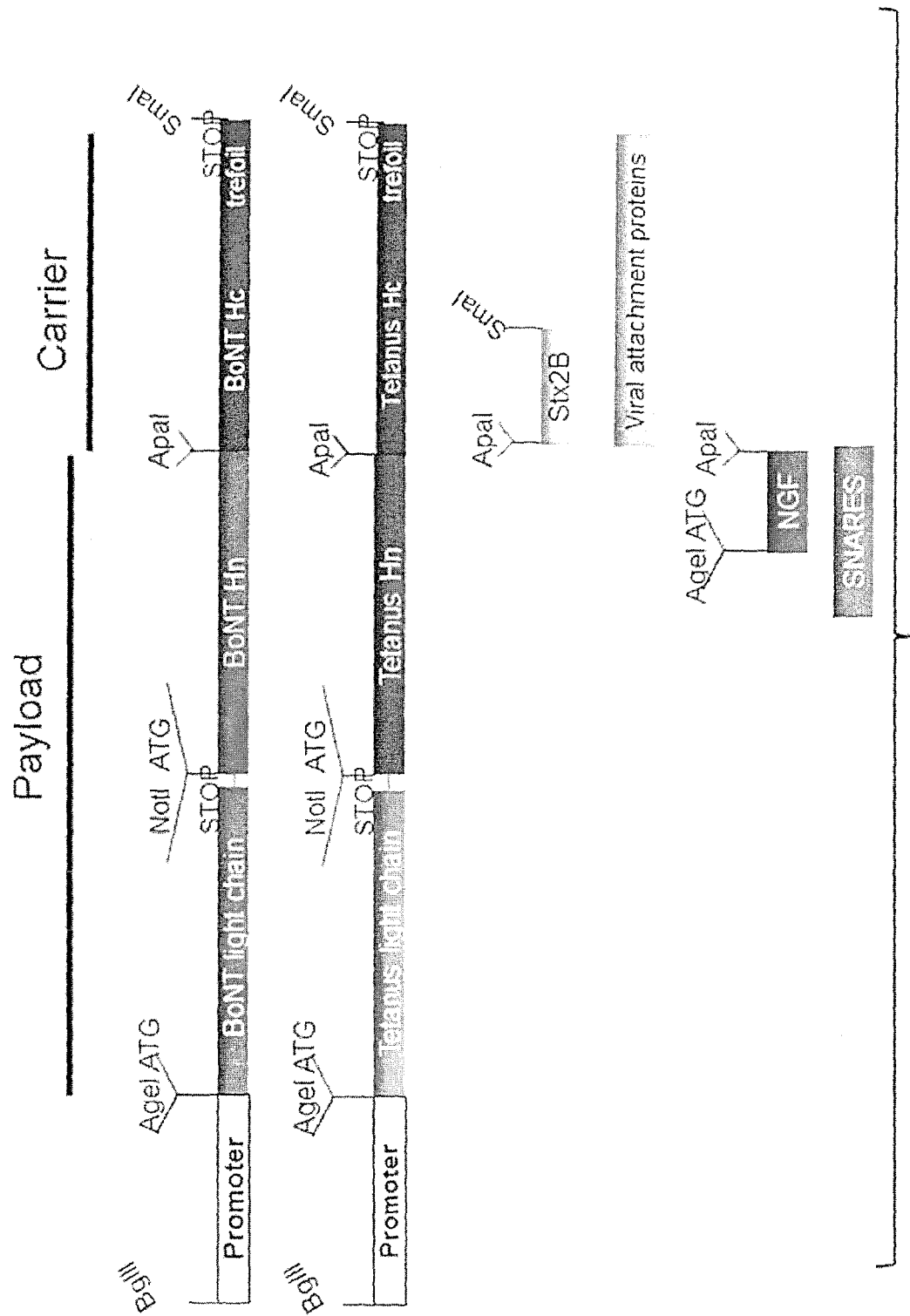


FIG. 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/041361**A. CLASSIFICATION OF SUBJECT MATTER****A61K 35/74(2006.01)i, A61K 35/66(2006.01)i, A61K 9/08(2006.01)i, A61K 9/06(2006.01)i, A61P 31/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/74; C07K 14/33; C12P 21/04; A61K 39/385; C12N 1/00; C12N 15/09; A61K 39/08; A61K 35/66; A61K 9/08; A61K 9/06; A61P 31/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: biological toxin, botulinum neurotoxin, Bacillus subtilis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6444209 B1 (JOHNSON, E. A. et al.) 03 September 2002 See claims 1 and 5; and columns 14-18.	1-70, 74-76
X	US 6967088 B1 (WILLIAMS, J. A. et al.) 22 November 2005 See abstract; and claims 1 and 2; and columns 16, 53 and 54.	1-70, 74-76
A	US 7189541 B2 (DONOVAN, S.) 13 March 2007 See abstract and claims 1-6.	1-70, 74-76
A	HILL, K. K. et al., 'Genetic diversity among botulinum neurotoxin-producing clostridial strains', Journal of Bacteriology, 2007, Vol. 189, No. 3, pages 818-832. See abstract and pages 819-821.	1-70, 74-76
A	US 2012-0245324 A1 (TON, J. L. et al.) 27 September 2012 See abstract and claims 1-6.	1-70, 74-76
A	US 2013-0129783 A1 (ALLERGAN, INC.) 23 May 2013 See abstract and claims 1-9.	1-70, 74-76



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 October 2014 (15.10.2014)

Date of mailing of the international search report

15 October 2014 (15.10.2014)

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INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/041361**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 71-73
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 71-73 pertain to a method for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/041361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6444209 B1	03/09/2002	US 05939070 A	17/08/1999
US 6967088 B1	22/11/2005	AU 1995-39683 B2	02/09/1999
		AU 1999-48763 A1	25/11/1999
		AU 1999-48763 B2	23/05/2002
		CA 2296765 A1	05/03/1998
		CA 2416318 A1	02/05/1996
		EP 0498854 A1	19/08/1992
		EP 0498854 B1	26/08/1998
		EP 0671902 A1	25/08/2004
		EP 0796326 A1	16/07/2003
		EP 1041149 A2	04/10/2000
		EP 1041149 A3	02/05/2001
		EP 1041149 B1	04/07/2007
		EP 1041149 B8	03/10/2007
		EP 1105153 A1	13/06/2001
		JP 2002-514886 A	21/05/2002
		JP 2003-137897 A	14/05/2003
		JP 3914484 B2	16/05/2007
		KR 10-0497700 B1	06/04/2006
		US 2003-0054493 A1	20/03/2003
		US 2003-0215468 A1	20/11/2003
		US 2003-0219457 A1	27/11/2003
		US 2004-0062771 A1	01/04/2004
		US 2004-0115215 A1	17/06/2004
		US 2004-0142455 A1	22/07/2004
		US 2004-0219637 A1	04/11/2004
		US 2004-0235118 A1	25/11/2004
		US 2004-0253673 A1	16/12/2004
		US 2005-0238665 A1	27/10/2005
		US 2007-0269861 A1	22/11/2007
		US 5196193 A	23/03/1993
		US 5340923 A	23/08/1994
		US 5443976 A	22/08/1995
		US 5466672 A	14/11/1995
		US 5599539 A	04/02/1997
		US 5601823 A	11/02/1997
		US 5719267 A	17/02/1998
		US 5736139 A	07/04/1998
		US 5762934 A	09/06/1998
		US 5814477 A	29/09/1998
		US 5904922 A	18/05/1999
		US 5919665 A	06/07/1999
		US 6290960 B1	18/09/2001
		US 6365158 B1	02/04/2002
		US 6573003 B2	03/06/2003
		US 6613326 B1	02/09/2003
		US 6613329 B1	02/09/2003
		US 6656468 B1	02/12/2003

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/041361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 7189541 B2	13/03/2007	WO 91-06306 A1	16/05/1991
		WO 94-13264 A1	23/06/1994
		WO 94-24155 A1	27/10/1994
		WO 96-12802 A1	02/05/1996
		WO 98-08540 A1	05/03/1998
		AR 045813 A1	16/11/2005
		AT 469215 T	15/06/2010
		AU 2004-280450 A1	21/04/2005
		AU 2004-280450 B2	05/02/2009
		AU 2005-293288 A1	20/04/2006
		AU 2005-293288 B2	02/06/2011
		AU 2005-327457 A1	05/10/2006
		AU 2005-327458 A1	05/10/2006
		AU 2005-327458 B2	15/12/2011
		AU 2006-295305 A1	05/04/2007
		BR PI0414722 A	21/11/2006
		CA 2540072 A1	21/04/2005
		CA 2556537 A1	03/09/2006
		CA 2556796 A1	03/09/2006
		CN 1856569 A	01/11/2006
		CN 1856569 B	01/12/2010
		CN 1930186 A	14/03/2007
		CN 1946738 A	11/04/2007
		DK 1664292 T3	30/08/2010
		EP 1396243 A1	10/03/2004
		EP 1396243 B1	15/08/2007
		EP 1664292 A2	07/06/2006
		EP 1664292 B1	26/05/2010
		EP 1827325 A1	05/09/2007
		EP 1827325 B1	04/05/2011
		EP 1853622 A1	14/11/2007
		EP 1853622 B1	16/07/2014
		EP 1853623 A1	14/11/2007
		EP 1853623 B1	08/05/2013
		EP 1924292 A2	28/05/2008
		EP 2177607 A1	21/04/2010
		EP 2335658 A1	22/06/2011
		ES 2344843 T3	08/09/2010
		JP 2007-506427 A	22/03/2007
		JP 2008-515582 A	15/05/2008
		JP 2008-531046 A	14/08/2008
		JP 2008-531047 A	14/08/2008
		JP 2009-508857 A	05/03/2009
		JP 2012-041356 A	01/03/2012
		JP 2012-070759 A	12/04/2012
		JP 2012-125251 A	05/07/2012
		JP 2013-014600 A	24/01/2013
		JP 4712807 B2	29/06/2011
		JP 4913074 B2	11/04/2012

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/041361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		JP 5006803 B2	22/08/2012
		JP 5122460 B2	16/01/2013
		JP 5518024 B2	11/06/2014
		JP 5567082 B2	06/08/2014
		KR 10-1100567 B1	29/12/2011
		KR 10-2006-0102330 A	27/09/2006
		KR 10-2007-0104334 A	25/10/2007
		KR 10-2007-0115580 A	06/12/2007
		KR 10-2007-0116710 A	11/12/2007
		NZ 545503 A	30/04/2009
		TW I294914 B	21/03/2008
		US 2005-0069562 A1	31/03/2005
		US 2005-0143765 A1	30/06/2005
		US 2005-0143766 A1	30/06/2005
		US 2005-0238668 A1	27/10/2005
		US 2005-0238669 A1	27/10/2005
		US 2005-0251182 A1	10/11/2005
		US 2006-0228777 A1	12/10/2006
		US 2006-0228780 A1	12/10/2006
		US 2006-0240514 A1	26/10/2006
		US 2007-0066541 A1	22/03/2007
		US 2007-0111288 A1	17/05/2007
		US 2008-0027469 A1	31/01/2008
		US 2009-0123497 A1	14/05/2009
		US 2009-0124790 A1	14/05/2009
		US 2009-0274729 A1	05/11/2009
		US 2010-0222587 A1	02/09/2010
		US 2011-0152608 A1	23/06/2011
		US 7148041 B2	12/12/2006
		US 7160699 B2	09/01/2007
		US 7238191 B2	03/07/2007
		US 7354740 B2	08/04/2008
		US 7445914 B2	04/11/2008
		US 7452697 B2	18/11/2008
		US 7560251 B2	14/07/2009
		US 7714024 B2	11/05/2010
		US 7901419 B2	08/03/2011
		US 7972346 B2	05/07/2011
		US 8008044 B2	30/08/2011
		US 8012162 B2	06/09/2011
		US 8012716 B2	06/09/2011
		US 8637571 B2	28/01/2014
		WO 2005-035749 A2	21/04/2005
		WO 2005-035749 A3	02/06/2005
		WO 2006-040647 A1	20/04/2006
		WO 2006-096163 A1	14/09/2006
		WO 2006-096164 A1	14/09/2006
		WO 2007-037849 A2	05/04/2007
		WO 2007-037849 A3	24/01/2008

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/041361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012-0245324 A1	27/09/2012	AU 2010-273601 A1	02/02/2012
		CA 2767760 A1	20/01/2011
		CN 102482332 A	30/05/2012
		EP 2454275 A1	23/05/2012
		JP 2012-532932 A	20/12/2012
		KR 10-2012-0105417 A	25/09/2012
		MX 2012000615 A	26/03/2012
		RU 2012104778 A	20/08/2013
		US 2012-0108792 A1	03/05/2012
		US 2012-0123095 A1	17/05/2012
		US 2012-0156756 A1	21/06/2012
		US 8129139 B2	06/03/2012
		US 8324349 B2	04/12/2012
		US 8357541 B2	22/01/2013
		WO 2011-0008713 A1	20/01/2011
US 2013-0129783 A1	23/05/2013	US 2004-0213811 A1	28/10/2004
		US 2008-0118592 A1	22/05/2008
		US 2010-0204127 A1	12/08/2010
		US 2012-0021991 A1	26/01/2012
		US 7393537 B2	01/07/2008
		US 7731977 B2	08/06/2010
		US 8057808 B2	15/11/2011
		US 8377452 B2	19/02/2013
		US 8747865 B2	10/06/2014