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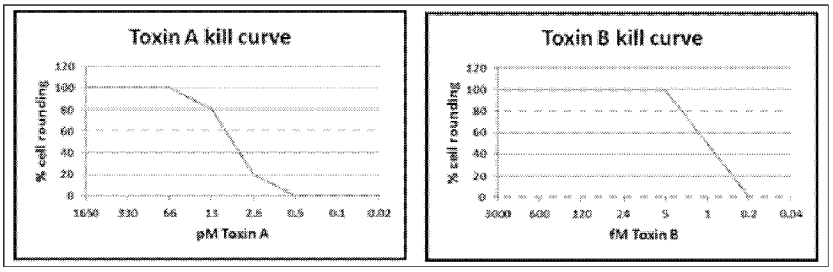
FIGURE 9



(57) Abstract: The present invention relates to, in part, methods and compositions for preventing and treating C. *difficile* infections and C. *difficile*-associated diseases. Particularly, pharmaceutical compositions comprising a recombinant yeast cell capable of producing at least one, optionally heteromultimeric, binding protein for neutralizing a C. difficile toxin are provided.

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METHODS AND COMPOSITIONS FOR INHIBITING CLOSTRIDIUM DIFFICILE

RELATED APPLICATIONS

[001] The present application claims priority to U.S. Provisional Application No. 62/074,993, filed November 4, 2014, the entire contents of which are incorporated by reference herein.

FIELD OF THE INVENTION

[002] The present invention relates to, in part, methods and compositions for preventing and treating *Clostridium difficile* infections (CDI) and *C. difficile*-associated diseases.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[003] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: SYN-006PC-SequenceListing; date recorded: November 4, 2015, 2015; file size: 123 KB).

BACKGROUND

[004] *Clostridium difficile* (*C. difficile*) is an anaerobic organism that poses significant health concern worldwide. *C. difficile* infection causes symptoms ranging from diarrhea to life-threatening inflammation of the colon (*e.g.*, pseudomembranous colitis). The organism cannot compete successfully with the normal microbial flora in the adult colon, but when the normal intestinal microflora is altered, for example by antibiotic treatment, *C. difficile* is able to colonize the gut in high numbers. Antibiotic therapy accounts for 98% of all cases of *C. difficile* associated diarrhea (CDAD). However, any predisposing condition which alters the normal intestinal flora, including any condition which requires extensive immunosuppressive treatment, can also lead to *C. difficile* infections and *C. difficile*-associated diseases.

[005] Pathogenic *C. difficile* strains excrete exotoxins toxin A and toxin B that have been linked to its pathogenicity. Both toxin A and toxin B are enterotoxic, capable of inducing intestinal epithelial cell damage and disrupting epithelium tight junctions leading to increased mucosal permeability. Moreover, these toxins induce production of immune mediators, leading to subsequent neutrophil infiltration and severe colitis.

[006] The primary treatment option for *C. difficile* infection includes antibiotics such as vancomycin or metronidazole, none of which is fully effective. Furthermore, a significant percentage of those infected with *C. difficile* relapse following treatment. Other options, such as probiotics, toxin-absorbing polymers and anion-exchange resins, have limited efficacy.

[007] There remains a need for safe and effective approaches for the treatment of *C. difficile* infections and *C. difficile*-associated diseases.

SUMMARY OF THE INVENTION

[008] Accordingly, the present invention provides compositions and methods for the treatment of *C. difficile* infections and *C. difficile*-associated diseases. Such compositions may comprise a recombinant yeast cell that is capable of producing, and optionally secreting, at least one, optionally heteromultimeric, binding protein, which is optionally a heavy-chain-only antibody (VHH), for neutralizing a *C. difficile* toxin (*e.g.* toxin A and/or toxin B). The optionally heteromultimeric neutralizing binding protein comprises two or more distinct binding regions, with each binding region binding to a non-overlapping portion of the *C. difficile* toxin. In some embodiments, the recombinant yeast cell is suitable for delivery of the anti-*C. difficile* agents to the GI tract.

[009] In various embodiments, the recombinant yeast cell is selected from *Saccharomyces spp.*, *Hansenula spp.*, *Kluyveromyces spp. Schizzosaccharomyces spp. Zygosaccharoinyces spp.*, *Pichia spp.*, *Monascus spp.*, *Geotrichum spp.* and *Yarrowia spp.* In some embodiments, the yeast cell is a *Saccharomyces cerevisiae*. In an embodiment, the yeast cell is a *Saccharomyces cerevisiae* subspecies *Saccharomyces boulardii*. In some embodiments, the yeast cell is *Pichia pastoris*. In some embodiments, the recombinant yeast cell is a probiotic.

[010] In various embodiments, the optionally heteromultimeric binding protein expressed by the yeast cell comprises a recombinant camelid heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', or a $F(ab')_2$. In one embodiment, the optionally heteromultimeric binding protein comprises a VHH. In some embodiments, the binding regions of the optionally heteromultimeric binding protein binds to *C. difficile* toxin A and/or toxin B and may comprise an amino acid sequence that is at least about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% identical to a sequence selected from SEQ ID NOs: 1-20, 41, 43, 45, 47, 49, 51, 54, and 56. In some embodiments, the binding regions of the optionally heteromultimeric binding protein comprise a nucleic acid sequence that is at least about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% identical to a nucleic acid sequence selected from SEQ ID NOs: 42, 44, 46, 48, 50, 52, 55, and 57. In some embodiments, the binding regions of the optionally heteromultimeric binding protein comprise a nucleic acid sequence that is a codon-optimized version of a nucleic acid sequence selected from SEQ ID NOs: 42, 44, 46, 48, 50, 52, 55, and 57.

[011] In various embodiments, the binding regions may be separated by a linker selected from a peptide or a protein. In an embodiment, the linker is a peptide having an amino acid sequence that is protease-resistant. In various embodiments, the optionally heteromultimeric binding protein further includes at least one tag that is an epitope recognized by an antibody. A replicable yeast expression vector comprising a nucleic acid encoding the optionally heteromultimeric neutralizing binding protein is also provided.

[012] Also provided are pharmaceutical compositions, which comprise the recombinant yeast cells described herein and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical

composition is formulated for intestinal delivery, for example, to the small intestine (*e.g.*, one or more of duodenum, jejunum, ileum) and/or the large intestine (*e.g.,* one or more of cecum, ascending, transverse, descending or sigmoid portions of the colon, and rectum). In some embodiments, the pharmaceutical composition is formulated for oral administration. In one embodiment, the pharmaceutical composition is formulated as a capsule or a tablet. In various embodiments, the pharmaceutical composition further includes additional therapeutic agents.

[013] In another aspect, the present invention provides use of the inventive pharmaceutical composition in treating or preventing *C. difficile* infections and *C. difficile*-associated diseases in a subject. The *C. difficile* infections and *C. difficile*-associated diseases may be *C. difficile* diarrhea (CDD), *C. difficile* intestinal inflammatory disease, colitis, pseudomembranous colitis, and/or peritonitis. In an embodiment, the *C. difficile* infection and/or *C. difficile*-associated disease is treated in the context of initial onset or relapse. In an embodiment, the subject is undergoing treatment or has recently undergone treatment with one or more primary antibiotic (*e.g.* metronidazole, vancomycin, *etc.*). In one embodiment, an initial and/or adjunctive therapy is administered to the subject. For example, the initial and/or adjunctive therapy may be one or more of metronidazole, vancomycin, fidaxomicin, rifaximin, fecal bacteriotherapy, probiotic therapy, and antibody therapy.

DESCRIPTION OF THE FIGURES

[014] **Figure 1** shows the amino acid sequence of four anti-*C. difficile*-toxin binding VHHs. Toxin A-specific VHHs are AA6 (SEQ ID NO: 2) and AH3 (SEQ ID NO: 1) and Toxin B-specific VHHS are E3 (SEQ ID NO: 12) and 5D (SEQ ID NO: 11). The VHHs are organized into four framework regions (FR1-4) and three antigen-binding regions (CDR1-3). The sequence derived from the J region overlaps with CDR3 and the FR4 region. The FR4-derived J regions are substituted. The putative Kex2 cleavage sites in 5D are designated in bold and underlined.

[015] **Figure 2** shows the amino acid sequence of the alpaca and llama joining regions (J regions). Substitution of the FR4 J4 region for the J7 region in llama VHHs resulted in a 100-fold increased secretion efficiency from S. *cerevisiae* but had no effect on secretion in HEK293 cells (Protein Engineering, Design & Selection vol. 25 no. 1 pp. 39–46, 2012, the entire contents of which are hereby incorporated by reference). The FR4 regions as indicated by arrows are identical. The amino acid changes in J7 compared to J4 are underlined. The following sequences are provided: YRYLEV (SEQ ID NO:23); NALDA (SEQ ID NO:24); EYDY (SEQ ID NO:25); DFGS (SEQ ID NO:26); YYGMDY (SEQ ID NO:27); SNSDYYD (SEQ ID NO:28); NQWRY (SEQ ID NO:29); YYGMDY (SEQ ID NO:30); WGQGTLVTVSS (SEQ ID NO:31); WGQGTQVTVSS (SEQ ID NO:32); and WGKGTLVTVSS (SEQ ID NO:33).

[016] **Figure 3** shows the amino acid sequence comparison of the Alpaca joining regions (J regions) of the four *C. difficile* anti-toxin VHHs and the Alpaca J4 region. The amino acid differences between the J4 region and the four *C. difficile* anti-toxin VHHs are underlined. The J sequences within the FR4 region are substituted to improve secretion in *S. cerevisiae*. The following sequences are provided: RGAV (SEQ ID NO:34);

RGPGTQVTVSS (SEQ ID NO:35); GKDD (SEQ ID NO:36); WGKGTLVTVSS (SEQ ID NO:33); GPEL (SEQ ID NO:37); RGQGIQVTVSS (SEQ ID NO:38); DYDV (SEQ ID NO:39); WGRGTQVAVSS (SEQ ID NO:40); EYDY (SEQ ID NO:25); and WGQGTQVTVSS (SEQ ID NO:32).

[017] **Figure 4** depicts SDS/PAGE analysis of the *C. difficile* anti-Toxin A VHH heterodimers AH3 + AA6 variants. Yeast cells were transformed with the indicated plasmids and yeast supernatants were denatured and analyzed by SDS/PAGE. "Neg" yeast that did not receive an expression plasmid. "Pos" yeast that were transformed with the 120955 Invertase-cutinase plasmid. The VHH transformed yeast secreted a ~38 kDa protein, indicated by the large arrow.

[018] **Figure 5** depicts Western analysis of *C. difficile* anti-Toxin A VHH heterodimers AH3 + AA6 variants. Western analysis was performed using an antibody specific to the 6-His tag. "Neg" yeast that did not receive an expression plasmid. "Pos" yeast that were transformed with the 120955 Invertase-cutinase plasmid. The ~38 kDa proteins contained the 6-His tag indicating they were the expressed VHH proteins.

[019] **Figure 6** depicts SDS/PAGE analysis of the *C. difficile* anti-Toxin B VHH heterodimers 5D + E3 variants. Yeast cells were transformed with the indicated plasmids and yeast supernatants were denatured and analyzed by SDS/PAGE. "Neg" yeast that did not receive an expression plasmid. "Pos" yeast that were transformed with the 120955 Invertase-cutinase plasmid. The VHH transformed yeast secreted a ~38 kDa protein, indicated by the large arrow.

[020] **Figure 7** depicts Western analysis of *C. difficile* anti-Toxin B VHH heterodimers 5D + E3 variants. Western analysis was performed using an antibody specific to the 6-His tag. "Neg" yeast that did not receive an expression plasmid. "Pos" yeast that were transformed with the 120955 Invertase-cutinase plasmid. The ~38 kDa proteins contained the 6-His tag indicating they were the expressed VHH proteins.

[021] **Figure 8** depicts SDS/PAGE of *C. difficile* anti-Toxin A and B VHH heterodimers variants. The highest expressing clone of the two clones analyzed per plasmid was analyzed by SDS/PAGE and compared to a standard curve from BSA dilutions. The bands were quantitated in comparison to the BSA standard by densitometry scanning.

[022] **Figure 9** depicts *C. difficile* Toxin A and Toxin B killing curves. The indicated dilutions of Toxin A or Toxin B were added to Vero cells and incubated for 24 hours. The percentage of cell rounding was monitored using a phase contrast microscope. For Toxin A 50 pM was used for subsequent assays and for Toxin B 40 fM was used.

[023] **Figure 10** depicts the results of a *C. difficile* Toxin A VHH neutralization assay. The indicated dilutions of the yeast supernatants containing the anti-Toxin A VHHs were added to Vero cells in 5-fold dilutions and incubated for 24 hours. The percentage of cell rounding was monitored using a phase contrast microscope.

[024] **Figure 11** depicts the results of a *C. difficile* Toxin B VHH neutralization assay. The indicated dilutions of the yeast supernatants containing the anti-Toxin B VHHs were added to Vero cells in 5-fold dilutions and incubated for 24 hours. The percentage of cell rounding was monitored using a phase contrast microscope.

DETAILED DESCRIPTION OF THE INVENTION

[025] The present invention is based, in part, on the surprising discovery that certain yeast cells are capable of producing and delivering at least one optionally heteromultimeric binding protein for neutralizing a *C. difficile* toxin and thus find use in the treatment of *C. difficile*-associated diseases. The present invention provides pharmaceutical compositions and their use for the treatment of *C. difficile* infections and *C. difficile*-associated diseases. The pharmaceutical composition may include a recombinant yeast cell that is capable of producing at least one optionally heteromultimeric binding protein for neutralizing a *C. difficile* toxin, such as, the *C. difficile* toxin A and/or toxin B. Such a composition delivers the therapeutic binding protein directly to the intestines for neutralizing *C. difficile* toxins, and provides a synergistic effect with other treatment modalities.

Binding Protein and Recombinant Yeast Cell

[026] In one aspect, the present invention provides a recombinant yeast cell that is capable of producing at least one binding protein for neutralizing a *C. difficile* toxin. In one embodiment, the binding protein neutralizes the *C. difficile* toxin A. In another embodiment, the binding protein neutralizes the *C. difficile* toxin B. In a further embodiment, the binding protein neutralizes both *C. difficile* toxin A and toxin B. In certain embodiments, the binding protein neutralizes or inhibits the *C. difficile* toxin(s) by preventing toxin entry into cells. In other embodiments, the binding protein neutralizes the toxin(s) and/or triggers an immune effector activity against the toxin(s).

[027] In one aspect, the present invention provides a recombinant yeast cell that is capable of producing at least one binding protein for neutralizing a *C. difficile* toxin (*e.g.* only A toxin, only B toxin, or both A and B toxin). In various embodiments, a cocktail of yeast may be used (*e.g.* a combination of yeast that are capable of producing A toxin or B toxin). In some embodiments, including cocktails or co-expression embodiments, the ratio of A versus B toxin may be varied (for example, the ratio of A:B may be about 0:100, or about 10:90, or about 20:80, or about 30:70, or about 40:60, or about 50:50, or about 60:40, or about 70:30, or about 80:20, or about 90:10, or about 100:0).

[028] In one embodiment, the binding protein is a monomer (*e.g.*, a single unit). For example, the binding protein has one binding region that binds to an epitope of the *C. difficile* toxin.

[029] Alternatively, the binding protein is a multimeric binding protein including two or more monomers such as, for example, a dimer, a trimer, a tetramer, a pentamer, an octamer, a 10-mer, a 15-mer, a 20-mer, or any multimer. Each monomer has a binding region that binds to an epitope of a disease agent (*e.g.*, the *C. difficile* toxin). In one embodiment, the multimeric binding protein includes the same monomer. Accordingly, the multimeric binding protein can be homogeneous and include two or more monomers having a binding region that

binds to the same site of a disease agent. In another embodiment, the multimeric binding protein includes different monomers (*i.e.*, heteromultimeric). Accordingly, the multimeric binding protein can be heterogeneous and include two or more monomers having binding regions that bind to two or more different sites of one or more disease agents. The optionally heteromultimeric binding protein can bind to non-overlapping portions of the molecular target and/or disease agent.

[030] In an illustrative embodiment, the binding protein is an optionally heteromultimeric binding protein including two or more binding regions, such that the binding regions are not identical and each binding region recognizes and binds a non-overlapping portion of the *C. difficile* toxin. For example, the binding regions may bind to non-overlapping portions of *C. difficile* toxin A or toxin B. Alternatively, the optionally heteromultimeric binding protein includes binding regions that bind specifically to different types of disease agents such as different types of pathogenic molecules such as bacteria, viruses, fungi, allergens, and toxins.

[031] In one embodiment, the optionally heteromultimeric neutralizing binding protein is heterodimeric. The heterodimeric binding protein includes a first binding region and a second binding region. In one embodiment, the first binding region and the second binding region both bind specifically to *C. difficile* toxin A. In another embodiment, the first and second binding regions both bind specifically to *C. difficile* toxin B. In a further embodiment, the first binding region binds specifically to *C. difficile* toxin A, and the second binding region binds specifically to *C. difficile* toxin B. In yet another embodiment, the binding protein may include one binding region that binds specifically to *C. difficile* toxin A and a second binding region that binds specifically to another disease agent such as, for example, a Shiga toxin.

[032] In another embodiment, the optionally heteromultimeric binding protein is heterotetrameric. In various embodiments, the heterotetrameric binding protein includes four binding regions that bind to either *C. difficile* toxin A, toxin B, or both toxin A and toxin B. Alternatively, the heterotetrameric binding protein may bind to *C. difficile* toxin A, toxin B, and/or another disease agent.

[033] In various embodiments, the binding protein comprises an intact antibody (*e.g.*, an intact monoclonal antibody) or an antigen-binding fragment of an antibody (*e.g.*, an antigen-binding fragment of a monoclonal antibody), including an intact antibody or antigen-binding fragment that has been modified, engineered or chemically conjugated. In certain embodiments, the binding protein comprises a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (also referred to as a cysteine knot protein or a knottin), a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, and/or a synthetic molecule.

[034] In one embodiment, the neutralizing binding protein comprises a VHH from, for example, an organism that produces VHH antibody such as a camelid, a shark, or a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH2

and CH3). VHHs are commercially available from ABLYNX INC. (Ghent, Belgium) under the trademark of NANOBODIES. In one embodiment, the neutralizing binding protein can be a VHH-based neutralizing agent (VNA). A VNA includes two or more VHH agents that each bind and neutralize one or more targets at non-overlapping sites. Illustrative VHH and VNA which neutralize *C. difficile* toxin A and/or toxin B are described for example, in U.S. Patent Publication No. US2013/0058962 and Yang, *et al.*, *J. Infect. Dis.* 210(6): 964 (2014), the entire contents of which are incorporated by reference herein.

[035] In various embodiments, the present invention contemplates the use of multimeric binding proteins having two or more monomers in which a monomer is exemplified by a VHH amino sequence. An embodiment of a multimeric binding protein includes two or more of a VHH sequences expressed as a single protein. Any combination of two or more of the VHH sequences can form a multimeric binding protein. In a particular embodiment, the present invention relates to a heterodimer, *i.e.*, protein, in which any two different VHH sequences herein are expressed as a single protein, *i.e.*, linked and expressed as a genetic fusion. Illustrative VHH sequences as described herein.

[036] In various embodiments, the binding protein is specific for *C. difficile* toxin A, and the binding region of the binding protein includes a recombinant VHH comprising an amino acid sequence selected from the following sequences, or variants thereof:

[037] AH3 (SEQ ID NO: 1)

QVQLVESGGGLVQPGGSLRLSCAASGFTLDYSSIGWFRQAPGKEREGVS
CISSSGDSTKYADSVKGRFTTSRDNAKNTVYLQMNSLKPDDTAVYYCAAF
RATMCGVFPLSPYGKDDWGKGTLVTVSSEPKTPKPQ

[038] AA6 (SEQ ID NO: 2)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSDYVMTWVRQAPGKGPEWIA
TINTDGSTMRDDSTKGRFTISRDNAKNTLYLQMTSLKPEDTALYYCARGRV
ISASAIRGAVRGPGTQVTVSSEPKTPKPQ

[039] A3H (SEQ ID NO: 3)

QVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIGWERQAPGKEREGVS
GISSVDGSTYYADSVRGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAD
QSPIPIHYSRTYSGPYGMDYWGKGTLVTVSSAHHSEDP;

[040] AC1 (SEQ ID NO: 4)

QLQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIGWERQAPGKEREGVS
GISFVDGSTYYADSVKGRFAISRGNAKNTVYLQMNSLKPEDTAVYYCAAD
QSSIPMHYSSTYSGPSGMDYWGKGTLVTVSSEPKTPKPQP

[041] A11G (SEQ ID NO: 5)

QLQLVETGGGLVQAGGSLRLSCAASGRTLSNYPMGWFRQAPGKEREFVA
AIRRIADGTYYADSVKGRFTISRDNAWNTLYLQMNGLKPEDTAVYFCATGP
GAFPGMVVTNPSAYPYWGQGTQVTVSSEPKTPKPQP

[042] AE1 (SEQ ID NO: 6)

QLQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREGVS
GISSSDGSTYYADSVKGRFTISRDNATNTVYLQMNSLKPEDTAVYYCAADQ
AAIPMHYSASYSGPRGMDYWGKGTLVTVSSEPKTPKPQP

[043] (SEQ ID NO: 7)

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQG
KLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFL
DANLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLG
TDDDDKAMAISDPNSQVQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIG
WFRQPPGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSL
KREDTAVYYCARRRFSASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQ
TSAIAGGGGSGGGGSGGGGSLQAMAAASQVQLVESGGGLVQTGGSLRL
SCASSGSIAGFETVTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISR
DNAKNTVYLQMNSLKPEDTGVYFCKGPELRGQGIQVTVSSEPKTPKPQPA
RR

[044] (SEQ ID NO: 8)

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQG
KLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFL
DANLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLG
TDDDDKAMAISDPNSQVQLVETGGLVQPGGSLRLSCAASGFTLDYSSIGW
FRQAPGKEREGVSCISSSGDSTKYADSVKGRETTSRDNAKNTVYLQMNSL
KPDDTAVYYCAAFRATMCGVFPLSPYGKDDWGKGTLVTVSSEPKTPKPQ
PTSAIAGGGGSGGGGSGGGGSLQAMAAAQLQLVETGGGLVQPGGSLRLS
CAASGFTFSDYVMTWVRQAPGKGPEWIATINTDGSTMRDDSTKGRFTISR
DNAKNTLYLQMTSLKPEDTALYYCARGRVISASAIRGAVRGPGTQVTVSSE
PKTPKPQPARQTSPSTVRLESRVRELEDRLEELRDELERAERRANEMSIQL
DEC

[045] In various embodiments, the binding protein is specific for *C. difficile* toxin B, and the binding region of the binding protein includes a recombinant VHH comprising an amino acid sequence selected from the following sequences, or variants thereof:

[046] 2D (SEQ ID NO:9)

QVQLVESGGGLVQPGGSLRLSCAASGFSLDYYGIGWFRQAPGKERQEVS
YISASAKTKLYSDSVKGRFTISRDNAKNAVYLEMNSLKREDTAVYYCARRR
FDASASNRWLAADYDYWGQGTQVTVSSEPKTPKPQ

[047] 20s (SEQ ID NO: 10)

QVQLVESGGGLVQAGGSLRLSCVSSERNPGINAMGWYRQAPGSQRKLVA
IWQTGGSLNYADSVKGRFTTSRDNLKNTVYLQMNSLKPEDTAVYYCYLKK
WRDQYWGQGTQVTVSSEPKTPKPQ

[048] 5D (SEQ ID NO: 11)

QLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQPPGKEREAVSYI
SASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKREDTAVYYCARRRFS
ASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQ

[049] E3 (SEQ ID NO: 12)

QVQLVESGGGLVQTGGSLRLSCASSGSIAGFETVTWSRQAPGKSLQWVAS
MTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMNSLKPEDTGVYFCKGPELRG
QGIQVTVSSEPKTPKPQ

[050] 7F (SEQ ID NO: 13)

QVQLVESGGGLVEAGGSLRLSCVVTGSSFSTSTMAWYRQPPGKQREWV
ASFTSGGAIKYTDSVKGRFTMSRDNAKKMTYLQMENLKPEDTAVYYCALH
NAVSGSSWGRGTQVTVSSEPKTPKPQ

[051] 5E (SEQ ID NO: 14)

VQLVESGGGLVQAGGSLRLSCAASGLMFGAMTMGWYRQAPGKEREMVA
YITAGGTESYSESVKGRFTISRINANNMVYLQMTNLKVEDTAVYYCNAHNF
WRTSRNWGQGTQVTVSSEPKTPKP

[052] B12 (SEQ ID NO: 15)

VQLVESGGGLVQAGDSLTLSCAASESTFNTFSMAWFRQAPGKEREYVAA
FSRSGGTTNYADSVKGRATISTDNAKNTVYLHMNSLKPEDTAVYFCAADR
PAGRAYFQSRSYNYWGQGTQVTVSSAHHSEDP

[053] A11 (SEQ ID NO: 16)

VQLVESGGGSVQIGGSLRLSCVASGFTFSKNIMSWARQAPGKGLEWVSTI
SIGGAATSYADSVKGRFTISRDNANDTLYLQMNNLKPEDTAVYYCSRGPRT
YINTASRGQGTQVTVSSEPKTPKP

[054] AB8 (SEQ ID NO: 17)

VQLVESGGGLVQAGGSLRLSCVGSGRNPGINAMGWYRQAPCiSQRELVA
VWQTGGSTNYADSVKGRFTISRDNLKNTVYLQMNSLKPEDTAVYYCYLKK
WRDEYWGQGTQVTVSSAHHSEDP

[055] C6 (SEQ ID NO: 18)

VQLVESGGGLVQAGESLRLSCVVSESIFRINTMGWYRQTPGKQREVVARI
TLRNSTTYADSVKGRFTISRDDAKNTLYLKMDSLKPEDTAVYYCHRYPMFR
NSPYWGQGTQVTVSSEPKTPKP

[056] C12 (SEQ ID NO: 19)

VQLVESGGGLVQAGESLRLSCVVSESIFRINTMGWYRQTPGKQREVVARI
TLRNSTTYADSVKGRFTISRDDAKNTITIXMDSIXPEDTAVYYCHRYPLIFRN
SPYWGQGTQVTVSSEPKTP

[057] A1 (SEQ ID NO: 20)

VQLVESGGGLVQAGGSLRLSCAAPGLTFTSYRMGWFRQAPGKEREYVAA
ITGAGATNYADSAKGRFTISKNNTASTVHLQMNSLKPEDTAVYYCAASNRA
GGYWRASQYDYWGQGTQVTVSSAHHSEDP

[058] In various embodiments, the amino acid sequence of the binding protein further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids as are known in the art.

[059] In various embodiments, the binding protein comprises a sequence that is at least about 60% identical to SEQ ID NOs: 1-20, 41, 43, 45, 47, 49, 51, 54, and 56. For example, the optionally heteromultimeric neutralizing binding protein may comprise a sequence that is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to SEQ ID NOs: 1-20, 41, 43, 45, 47, 49, 51, 54, and 56.

[060] In certain embodiments, the binding protein comprises a sequence that includes at least one amino acid alteration with respect to SEQ ID NOs: 1-20, 41, 43, 45, 47, 49, 51, 54, and 56. For example, the binding protein comprises a sequence that includes at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 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, 40, 49, or 50 amino acid alterations with respect to SEQ ID NOs: 1-20, 41, 43, 45, 47, 49, 51, 54, and 56. The amino acid alteration can be an amino acid deletion, insertion, substitution, or modification. In one embodiment, the amino acid alteration is an amino acid substitution.

[061] In various embodiments, the amino acid alteration may be in the Complementarity Determining Regions (CDRs) of the binding protein (*e.g.*, the CDR1, CDR2 or CDR3 regions). In another embodiment, amino acid alteration may be in the framework regions (FRs) of the binding protein (*e.g.*, the FR1, FR2, FR3, or FR4 regions). In a further embodiment, the amino acid alteration may be in the joining regions (J regions) of the binding protein (*e.g.*, the J1, J2, J3, J4, J5, J6, or J7 regions).

[062] Modification of the amino acid sequence of recombinant binding protein is achieved using any known technique in the art *e.g.*, site-directed mutagenesis or PCR based mutagenesis. Such techniques are described, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Plainview, N.Y., 1989 and Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y., 1989.

[063] In certain embodiments, the binding protein includes a linker that connects each binding region and/or subunits of the multimeric protein. For example, the linker is a single amino acid or a plurality of amino acids that does not affect or reduce the stability, orientation, binding, neutralization, and/or clearance characteristics of the binding regions and binding protein.

[064] The linker can be non-functional or functional. "Non-functional" refers to non-reactive hydrocarbon chains, simple amino acid sequences, or other sequences that simply bind covalently to the binding regions. A "functional linker" can comprise amino acid residues that confer biological properties useful for imaging, diagnostics, therapy, *etc.* Such a functionality can include peptide or protein binding motifs, protein kinase consensus sequences, protein phosphatase consensus sequences, or protease-reactive or protease-specific sequences. For example, the linker may include the amino acid sequence GGGGS (SEQ ID NO: 21). In another example, the linker may include the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 22) or a portion thereof. In a related embodiment, the linker is a flexible linker located within subunits/domains of the binding protein, such that the linker does not negatively affect the function of the binding protein in neutralizing the *C. difficile* toxin. The linker in various embodiments stabilizes the binding protein and does not prevent the respective binding of the binding regions to the *C. difficile* toxin or toxins. For example the linker may include amino acid sequences/residues including serine and glycine, and in various embodiments is at least about three to five amino acids long, or about five to eight amino acids long, or about eight to fifteen amino acids long.

[065] In certain embodiments, the binding protein includes at least one tag that is attached or genetically fused to the binding protein. The binding protein may include a single tag or multiple tags. In an embodiment, the binding protein is multimeric and has a single tag on each monomer or has two or more tags on each monomer. Alternatively, the binding protein comprises no tags attached to the monomers and/or linker. The tag for example is a peptide, sugar, or DNA molecule that does not inhibit or prevent binding of the binding protein and/or binding regions to the *C. difficile* toxin or toxins. In various embodiments, the tag is at least about: three to five amino acids long, five to eight amino acids long, eight to twelve amino acids long, twelve to fifteen amino acids long, or

fifteen to twenty amino acids long. Illustrative tags are described for example, in U.S. Patent Publication No. US2013/0058962, the contents of which are hereby incorporated by reference in their entirety.

[066] In certain embodiments, presence of the tag on or operably fused to the binding protein and/or binding region synergistically induces clearance of the disease agent (*e.g.*, the *C. difficile* toxin) from the body. For example the tag attached to the binding protein induces an immune response from a patient or subject contacted with a pharmaceutical composition containing the tagged-binding protein. In certain embodiments the tag includes a portion (*e.g.*, conserved, unique, in-activated, and non-functional) of a pathogenic molecule. In certain embodiments, the tag is an adjuvant. *See* Gerber *et al.* U.S. Pat. No. 7,879,333, which is incorporated by reference herein in its entirety. In various embodiments, the tag is an epitope that is recognized by an antibody. For example, the tag is a peptide, carbohydrate, polymer, or nucleic acid that is effective for enhancing neutralization and/or clearance of the disease agent or plurality of disease agents.

[067] The invention also provides for polynucleotides encoding any of the binding proteins of the invention, including, for example, replicable yeast expression vectors comprising such polynucleotides. Such polynucleotides may further comprise, in addition to sequences encoding the binding proteins of the invention, one or more expression control elements. For example, the polynucleotide, may comprise one or more promoters or transcriptional enhancers, ribosomal binding sites, transcription termination signals, and polyadenylation signals, as expression control elements. In addition, the polynucleotides may include, for example, leader sequences to facilitate secretion of the binding protein from yeast cells. Illustrative secretion signal sequences, include, but are not limited to, alpha-factor full, alpha-factor, alpha-amylase, glucoamylase, inulinase, invertase, killer protein, lysozyme, and serum albumin sequences.

[068] The present invention contemplates expression of the binding protein in a recombinant yeast cell. The recombinant yeast cell may be generated by stable integration into yeast chromosomal DNA of expression cassette(s) that encode and can express the one or more binding proteins. Alternatively, recombinant yeast cell may be generated using a process in which the yeast maintains an expression cassette(s) that encode and can express the one or more binding proteins on a stable episome. The recombinant yeast cell may be any yeast cell that is capable of surviving in the mammalian intestine. In various embodiments, the yeast cell has a known probiotic capacity, such as yeast strains selected from kefir, kombucha or dairy products. In certain embodiments, the recombinant yeast cell is selected from *Saccharomyces spp.*, *Hansenula spp.*, *Kluyveromyces spp. Schizzosaccharomyces spp. Zygosaccharomyces spp.*, *Pichia spp.*, *Monascus spp.*, *Paffia spp.*, *Candida spp., Talaromyces spp., Brettanomyces spp., Pachysolen spp., Debaryomyces spp., Geotrichum spp.* and *Yarrowia spp.* In one embodiment, the recombinant yeast cell is *Saccharomyces cerevisiae*. In another embodiment, the recombinant yeast cell is the *Saccharomyces cerevisiae* subspecies *Saccharomyces boulardii* (by way of non-limiting example, ATCC 74352 and/or any cells in US Patent Nos. 6,010,695 and 7,799,328 the contents of which are hereby incorporated by reference in their entirety). In some embodiments, the yeast cell is *Pichia pastoris* (by way of non-limiting example, the *Pichia pastoris* strains NRRL Y-11430, NRRL Y-11431,

GS115 (NRRL Y-15851), GS190 (NRRL Y-18014) disclosed in U.S. Pat. No. 4,818,700, PPF1 (NRRL Y-18017) disclosed in U.S. Pat. No. 4,812,40, and any cells disclosed in U.S. Patent Nos. 5,330,901 and 7,442,371).

Pharmaceutical Compositions

[069] The present invention provides a pharmaceutical composition for use in, for example, the treatment of *C. difficile* infections and *C. difficile*-associated diseases. The pharmaceutical composition comprises a recombinant yeast cell capable of producing at least one binding protein for neutralizing a *C. difficile* toxin as described herein.

[070] In various embodiments, the present invention pertains to pharmaceutical compositions comprising a recombinant yeast cell described herein and a pharmaceutically acceptable carrier or excipient as described herein. In a specific embodiment, the pharmaceutical composition is formulated for oral administration, *e.g.* as a tablet or multi-particulate sprinkle, a capsule, or a gelatin capsule.

[071] However, as described herein, other administration routes and formulations are also provided.

[072] In some embodiments, the pharmaceutical compositions may be used in conjunction with or be co-formulations with an additional agent. In some embodiments, the additional agent is an additional antibiotic degradation enzyme, such as, for example, a beta-lactamase, such as a beta-lactamase described in US Patent Publication No. 2014/0127785, the entire contents of which are incorporated herein in its entirety. In some embodiments, the antibiotic degradation enzyme is selected from a functional Group 1, Group 2, Group 3, or a Group 4 beta-lactamase (*see*, *e.g.*, Bush *et al.*, *Antimicrob. Agents Chemother*, 39: 1211, the contents of which are hereby incorporated by reference) and/or a molecular/Ambler class A, or class B, or class C, or class D beta-lactamase (*see*, *e.g.*, Ambler 1980, *Philos Trans R Soc Lond B Biol Sci.* 289: 321 the contents of which are hereby incorporated by reference). In some embodiments, the antibiotic degradation enzyme is a serine beta-lactamase or a zinc-dependent (EDTA-inhibited) beta-lactamase. For example, in some embodiments, the beta-lactamase is one or more of P1A, P2A, or P3A. Further, the beta-lactamase may be an extended-spectrum beta-lactamase (ESBL), optionally selected from a TEM, SHV, CTX-M, OXA, PER, VEB, GES, and IBC beta-lactamase. Further, the beta-lactamase may be an inhibitor-resistant β-lactamase, optionally selected from an AmpC-type β-lactamases, Carbapenemase, IMP-type carbapenemases (metallo-β-lactamases), VIM (Verona integron-encoded metallo-β-lactamase), OXA (oxacillinase) group of β-lactamases, KPC (*K. pneumonia* carbapenemase), CMY (Class C), SME, IMI, NMC and CcrA, and a NDM (New Delhi metallo-β-lactamase, *e.g.* NDM-1) beta-lactamase.

[073] In some embodiments, the additional agent is an adjunctive therapy that is used in, for example, the treatment of CDI. In some embodiments, the additional agent is metronidazole (*e.g.* FLAGYL), fidaxomicin (*e.g.* DIFICID), or vancomycin (*e.g.* Vancocin, rifaximin, fecal bacteriotherapy, charcoal-based binders (*e.g.* DAV132), probiotic therapy (*see*, *e.g.*, *Intnat'l J Inf Dis*, 16 (11): e786, the contents of which are hereby incorporated by reference, illustrative probiotics include *Lactobacillus rhamnosus* GG; *Lactobacillus plantarum* 299v; *Clostridium butyricum* M588; *Clostridium difficile* VP20621 (non-toxigenic *C. difficile* strain);

combination of *Lactobacillus casei*, *Lactobacillus acidophilus* (Bio-K + CL1285); combination of *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus* (Actimel); combination of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* (Florajen3); combination of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus delbrueckii* subsp. *bulgaricus*, *Lactobacillus bulgaricus casei*, *Lactobacillus bulgaricus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Streptococcus salivarius* subsp. *thermophilus* (VSL#3)) and antibody or other biologic therapy (*e.g.* monoclonal antibodies against *C. difficile* toxins A and B as described in *N Engl J Med.* 2010;362(3):197, the content of which are hereby incorporated by reference in their entirety; or any neutralizing binding protein directed against *C. difficile* binary toxin. In some embodiments, various penicillins and cephalosporins may be the additional agent.

[074] For all additional agent compositions, targeting to various parts of the GI tract may be employed as described herein.

[075] Any pharmaceutical compositions (and/or additional agents) described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration.

[076] Pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents. Other examples of suitable pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

[077] The present invention includes the described pharmaceutical compositions (and/or additional agents) in various formulations. Any inventive pharmaceutical composition (and/or additional agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, gelatin capsules, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, cryopreserved yeast, lyophilized powder, frozen suspension, dessicated powder, or any other form suitable for use. In some embodiments, the pharmaceutical compositions provide for live, viable yeast (*e.g.* more than 10% of yeast are viable, or more than 10% of yeast are viable, or more than 20% of yeast are

viable, or more than 30% of yeast are viable, or more than 40% of yeast are viable, or more than 50% of yeast are viable, or more than 60% of yeast are viable, or more than 70% of yeast are viable, or more than 80% of yeast are viable, or more than 90% of yeast are viable, or about 100% of yeast are viable). In one embodiment, the composition is in the form of a capsule (*see, e.g.*, U.S. Patent No. 5,698,155). In another embodiment, the composition is in the form of a tablet. In yet another embodiment, the pharmaceutical composition is formulated in the form of a soft-gel capsule. In a further embodiment, the pharmaceutical composition is formulated in the form of a gelatin capsule. In yet another embodiment, the pharmaceutical composition is formulated as a liquid.

[078] Where necessary, the inventive pharmaceutical compositions (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device.

[079] The formulations comprising the inventive pharmaceutical compositions (and/or additional agents) of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the therapeutic agents into association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation (*e.g.*, wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art).

[080] In one embodiment, any pharmaceutical compositions (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

[081] In various embodiments, the administration of any inventive pharmaceutical compositions (and/or additional agents) is any one of oral, intravenous, and parenteral. In various embodiments, the administration of any inventive pharmaceutical compositions comprising a yeast cell is into the GI tract via, for example, oral delivery, nasogastral tube, intestinal intubation (*e.g.* an enteral tube or feeding tube such as, for example, a jejunal tube or gastro-jejunal tube, etc.), endoscopy, colonoscopy, or enema. In one embodiment, any pharmaceutical compositions (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for oral administration.

[082] Compositions for oral delivery can be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, sprinkles, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions can comprise one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration to provide a sustained action over an extended period of

time. Selectively permeable membranes surrounding an osmotically active agent driving any inventive pharmaceutical compositions (and/or additional agents) described herein are also suitable for orally administered compositions. In these latter platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time-delay material such as glycerol monostearate or glycerol stearate can also be useful. Oral compositions can include standard excipients such as mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, ethacrylic acid and derivative polymers thereof, and magnesium carbonate. In one embodiment, the excipients are of pharmaceutical grade. Suspensions, in addition to the active compounds, may contain suspending agents such as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, *etc.*, and mixtures thereof.

[083] Inventive pharmaceutical compositions (and/or additional agents) may be administered to a subject, by, for example, directly or indirectly contacting the mucosal tissues of the gastrointestinal tract. The gastrointestinal tract includes organs of the digestive system such as mouth, esophagus, stomach, duodenum, small intestine, large intestine and rectum and includes all subsections thereof (*e.g.* the small intestine may include the duodenum, jejunum and ileum; the large intestine may include the colon transversum, colon descendens, colon ascendens, colon sigmoidenum and cecum). Various methods may be used to formulate and/or deliver the agents descried herein to a location of interest. For example, the inventive pharmaceutical compositions (and/or additional agents) described herein may be formulated for delivery to one or more of the stomach, small intestine, large intestine and rectum and includes all subsections thereof (*e.g.* duodenum, jejunum and ileum, colon transversum, colon descendens, colon ascendens, colon sigmoidenum and cecum). In some embodiments, the compositions described herein may be formulated to deliver to the upper or lower GI tract.

[084] In some embodiments, the compositions of the invention are formulated for enteric delivery. For example, the compositions may be formulated as capsules or tablets for oral delivery, and may comprise a delayed-release coating containing one or more enteric agents. A delayed-release coating is substantially stable in gastric fluid and substantially unstable (*e.g.*, dissolves rapidly or is physically unstable) in intestinal fluid, thus providing for substantial release of the active agent from the composition in the affected region of the small intestine, *e.g.*, the duodenum, the jejunum, and/or the ileum or large intestine, *e.g.* the colon transversum, colon descendens, colon ascendens, colon sigmoidenum and cecum.

[085] The inventive pharmaceutical compositions (and/or additional agents) are generally stable in gastric fluid or simulated intestinal fluid, that is, the compositions are stable in acidic environments. Thus, the compositions release less than 30% by weight of the active agent in gastric fluid with a pH of 5 or less, or simulated gastric fluid with a pH of 5 or less, in approximately 120 minutes. Compositions of the invention may release from about 0% to about 25%, or from about 0% to about 10% by weight of the active agent in gastric fluid

with a pH of 5 or less, or simulated gastric fluid with a pH of 5 or less, in approximately 120 minutes. Compositions of the invention in certain embodiments release no more than about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, or about 10% by weight of the active agent in gastric fluid, or simulated gastric fluid with a pH of 5 or less, in approximately 120 minutes. The pharmaceutical compositions generally release active agent to act locally at regions of the small or large intestine. In certain embodiments, the composition of the invention release about 70% or more by weight of the active agent in the small or large intestine within about 120 minutes. In certain embodiments, the composition releases 80% or more, or 90% or more, of the active agent in small or large intestine, within about ninety minutes or within about 120 minutes. In certain embodiments this release in the small or large intestine is mediated by pH of gastric fluid or simulated gastric fluid-for example, a release when the pH of about 5 or greater (*e.g.* pH of about 5.5–6.5 for release in the duodenum, pH of about 6-7 for release in the colon ascendens or jejunum, pH of about 6.5–7 for release in the ileum, pH of about 7-7.5 for release in the colon descendens).

[086] Examples of simulated gastric fluid and simulated intestinal fluid include, but are not limited to, those disclosed in the 2005 Pharmacopeia 23NF/28USP in Test Solutions at page 2858 and/or other simulated gastric fluids and simulated intestinal fluids known to those of skill in the art, for example, simulated gastric fluid and/or intestinal fluid prepared without enzymes.

[087] The pharmaceutical composition may control intestinal release of the active agent through one or more delayed-release coating(s), which remain essentially intact, and/or which may be essentially insoluble, in gastric fluid. The stability of the delayed-release coating can be pH dependent. Delayed-release coatings that are pH dependent will be substantially stable in acidic environments (pH 5 or less), and substantially unstable in near neutral to alkaline environments (pH greater than 5). For example, the delayed-release coating may essentially disintegrate or dissolve in near neutral to alkaline environments, such as are found in the small intestine or large intestine, to thereby release active agent locally to diseased or affected tissue.

[088] Alternatively, the stability of the delayed-release coating may be enzyme-dependent. Delayed-release coatings that are enzyme dependent will be substantially stable in fluid that does not contain a particular enzyme and substantially unstable in fluid containing the enzyme. The delayed-release coating will essentially disintegrate or dissolve in fluid containing the appropriate enzyme. Enzyme-dependent control can be brought about, for example, by using materials which release the active ingredient only on exposure to enzymes in the intestine, such as galactomannans. Accordingly, in one embodiment, the delayed-release coating may be degraded by a microbial enzyme present in the gut flora. In one embodiment, the delayed-release coating may be degraded by a bacteria present in the small intestine. In another embodiment, the delayed-release coating may be degraded by a bacteria present in the large intestine.

[089] In some embodiments, the target organ for the release of the active agent from the compositions of the invention, which include gastric resistant capsules or tablets, is the small intestine, *e.g.*, the ileum, the duodenum and the jejunum or the large intestine, *e.g.* the colon transversum, colon descendens, colon

ascendens, colon sigmoidenum and cecum. *See* Remington's Pharmaceutical Sciences, 16th Ed., Eds. Osol, Mack Publishing Co., Chapter 89 (1980); Digenis *et al.*, *J. Pharm. Sci.*, 83:915-921 (1994); Vantini *et al.*, *Clinica Terapeutica*, 145:445-451 (1993); Yoshitomi *et al.*, *Chem. Pharm. Bull.*, 40:1902-1905 (1992); Thoma *et al.*, *Pharmazie*, 46:331-336 (1991); Morishita *et al.*, *Drug Design and Delivery*, 7:309-319 (1991); and Lin *et al.*, *Pharmaceutical Res.*, 8:919-924 (1991) for examples of the preparation of such tablets or capsules (the contents of all of which are hereby incorporated by reference in their entireties).

[090] In some embodiments, the compositions of the present invention may be formulated using the EUDRAGIT system, as known in the art and described in *Pharma Polymer* No. 7, Oct. 2000, the contents of which are hereby incorporated by reference in their entirety. The Eudragit-type polymer include, for example, Eudragit® RL 30 D, RL PO, RL 100, RL 12,5, RS 30 D, RS Po, RS 100, RS 12,5, NE 30 D, NE 40 D and NM 30 D.

[091] Any inventive pharmaceutical compositions (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropyl cellulose, hydropropylmethyl cellulose, polyvinylpyrrolidone, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Suitable controlled- or sustained-release formulations known to those skilled in the art, including those described herein, can be readily selected for use with the active ingredients of the agents described herein. The invention thus provides single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled- or sustained-release.

[092] Controlled- or sustained-release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

[093] In another embodiment, a controlled-release system can be placed in proximity of the target area to be treated, thus requiring only a fraction of the systemic dose (s*ee, e.g.,* Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, *Science* 249:1527-1533) may be used.

[094] Accordingly, in one embodiment, the pharmaceutical composition is formulated to release the recombinant yeast cell in the intestines. In various embodiments, the pharmaceutical composition of the present invention releases at least 60% of the recombinant yeast cell in the intestine. For example, the pharmaceutical

composition releases at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the recombinant yeast cell in the intestine.

[095] In various embodiments, the pharmaceutical composition is formulated to release the recombinant yeast cell in the small intestine. In one embodiment, the pharmaceutical composition releases the recombinant yeast cell in the duodenum. In another embodiment, the pharmaceutical composition releases the recombinant yeast cell in the jejunum. In a further embodiment, the pharmaceutical composition releases the recombinant yeast cell in the ileum.

[096] In other embodiments, the pharmaceutical composition releases the recombinant yeast cell in the large intestine. In an embodiment, the pharmaceutical composition releases the recombinant yeast cell in the cecum. In another embodiment, the pharmaceutical composition releases the recombinant yeast cell in the ascending colon. In yet another embodiment, the recombinant yeast cell is released in the transverse colon. In a further embodiment, the recombinant yeast cell is released in the descending colon. In another embodiment, the recombinant yeast cell is released in the sigmoid colon.

[097] In certain embodiments, the recombinant yeast cell is not substantially released in the stomach.

[098] In various embodiments, the pharmaceutical composition is formulated so as to deliver viable recombinant yeast cells to the intestines where active binding proteins are secreted by the yeast cells to neutralize a *C. difficile* toxin. In one embodiment, the pharmaceutical composition is formulated as an enteric-coated capsule which directly releases the recombinant yeast cell in the intestines. In other embodiments, the pharmaceutical composition can be formulated as a gelatin capsule, or the recombinant yeast cell can be dissolved in a liquid and ingested. In such embodiments, the recombinant yeast cell is delivered anywhere along the GI tract. The recombinant yeast cell, for example, *Saccharomyces boulardii* or *Pichia pastoris*, is resistant to stomach acid and remains viable during transit to the intestine, where it secretes active binding proteins for neutralizing the *C. difficile* toxin.

Administration and Dosage

[099] It will be appreciated that the actual dose of the recombinant yeast cell to be administered according to the present invention will vary according to the particular dosage form, and the mode of administration. Many factors that may modify the action of the recombinant yeast cell (*e.g.*, body weight, gender, diet, time of administration, route of administration, rate of excretion, condition of the subject, drug combinations, genetic disposition and reaction sensitivities) can be taken into account by those skilled in the art. Administration can be carried out continuously or in one or more discrete doses within the maximum tolerated dose. Optimal

administration rates for a given set of conditions can be ascertained by those skilled in the art using conventional dosage administration tests.

[0100] In some embodiments, a suitable dosage of the recombinant yeast cell is in a range of about 0.01 mg/kg to about 10 g/kg of body weight of the subject, about 0.01 mg/kg to about 1 g/kg of body weight of the subject, about 0.01 mg/kg to about 100 mg/kg of body weight of the subject, about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, about 100 mg/kg body weight, about 1 g/kg of body weight, about 10 g/kg of body weight, inclusive of all values and ranges therebetween.

[0101] Individual doses of the recombinant yeast cell can be administered in unit dosage forms (*e.g.*, tablets or capsules) containing, for example, from about 0.01 mg to about 100 g, from about 0.01 mg to about 75 g, from about 0.01 mg to about 50 g, from about 0.01 mg to about 25 g, about 0.01 mg to about 10 g, about 0.01 mg to about 7.5 g, about 0.01 mg to about 5 g, about 0.01 mg to about 2.5 g, about 0.01 mg to about 1 g, about 0.01 mg to about 100 mg, from about 0.1 mg to about 100 mg, from about 0.1 mg to about 90 mg, from about 0.1 mg to about 80 mg, from about 0.1 mg to about 70 mg, from about 0.1 mg to about 60 mg, from about 0.1 mg to about 50 mg, from about 0.1 mg to about 40 mg active ingredient, from about 0.1 mg to about 30 mg, from about 0.1 mg to about 20 mg, from about 0.1 mg to about 10 mg, from about 0.1 mg to about 5 mg, from about 0.1 mg to about 3 mg, from about 0.1 mg to about 1 mg per unit dosage form, or from about 5 mg to about 80 mg per unit dosage form. For example, a unit dosage form can be about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1g, about 2.5 g, about 5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

[0102] In one embodiment, the recombinant yeast cell is administered at an amount of from about 0.01 mg to about 100 g daily, from about 0.01 mg to about 75 g daily, from about 0.01 mg to about 50 g daily, from about 0.01 mg to about 25 g daily, from about 0.01 mg to about 10 g daily, from about 0.01 mg to about 7.5 g daily, from about 0.01 mg to about 5 g daily, from about 0.01 mg to about 2.5 g daily, from about 0.01 mg to about 1 g daily, from about 0.01 mg to about 100 mg daily, from about 0.1 mg to about 100 mg daily, from about 0.1 mg to

about 95 mg daily, from about 0.1 mg to about 90 mg daily, from about 0.1 mg to about 85 mg daily, from about 0.1 mg to about 80 mg daily, from about 0.1 mg to about 75 mg daily, from about 0.1 mg to about 70 mg daily, from about 0.1 mg to about 65 mg daily, from about 0.1 mg to about 60 mg daily, from about 0.1 mg to about 55 mg daily, from about 0.1 mg to about 50 mg daily, from about 0.1 mg to about 45 mg daily, from about 0.1 mg to about 40 mg daily, from about 0.1 mg to about 35 mg daily, from about 0.1 mg to about 30 mg daily, from about 0.1 mg to about 25 mg daily, from about 0.1 mg to about 20 mg daily, from about 0.1 mg to about 15 mg daily, from about 0.1 mg to about 10 mg daily, from about 0.1 mg to about 5 mg daily, from about 0.1 mg to about 3 mg daily, from about 0.1 mg to about 1 mg daily, or from about 5 mg to about 80 mg daily. In various embodiments, the recombinant yeast cell is administered at a daily dose of about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 7.5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

[0103] In some embodiments, the recombinant yeast cell secretes a therapeutically effective amount of one or more binding proteins. In some embodiments, the recombinant yeast cell secretes an amount of one or more binding proteins that results in intestinal fluid levels of about 1 pg/mL, or about 1 ng/mL, 1 µg/ml, or about 1 mg/mL. In some embodiments the yeast secrete an amount of one or more binding proteins that results in intestinal fluid levels of about 10 ng/mL, or about 100 ng/mL, or about 1 µg/mL, or about 10 µg/mL, or about 100 µg/mL.

[0104] In accordance with certain embodiments of the invention, the pharmaceutical composition comprising the recombinant yeast cell may be administered, for example, more than once daily (*e.g.*, about two times, about three times, about four times, about five times, about six times, about seven times, about eight times, about nine times, or about ten times daily), about once per day, about every other day, about every third day, about once a week, about once every two weeks, about once every month, about once every two months, about once every three months, about once every six months, or about once every year.

[0105] In various embodiments, the pharmaceutical composition of the present invention is co-administered in conjunction with additional agent(s) described herein. Co-administration can be simultaneous or sequential.

[0106] In one embodiment, the additional agent and the recombinant yeast cell of the present invention are administered to a subject simultaneously. The term “simultaneously” as used herein, means that the additional agent and the recombinant yeast cell are administered with a time separation of no more than about

60 minutes, such as no more than about 30 minutes, no more than about 20 minutes, no more than about 10 minutes, no more than about 5 minutes, or no more than about 1 minute. Administration of the additional agent and the recombinant yeast cell can be by simultaneous administration of a single formulation (*e.g.*, a formulation comprising the additional agent and the recombinant yeast cell) or of separate formulations (*e.g.*, a first formulation including the additional agent and a second formulation including the recombinant yeast cell). For example, in one embodiment, the recombinant yeast cell is administered simultaneously with an antibiotic. In another embodiment, the recombinant yeast cell is administered simultaneously with a beta-lactamase.

[0107] Co-administration does not require the therapeutic agents to be administered simultaneously, if the timing of their administration is such that the pharmacological activities of the additional agent and the recombinant yeast cell overlap in time, thereby exerting a combined therapeutic effect. For example, the additional agent and the recombinant yeast cell can be administered sequentially. The term "sequentially" as used herein means that the additional agent and the recombinant yeast cell are administered with a time separation of more than about 60 minutes. For example, the time between the sequential administration of the additional agent and the recombinant yeast cell can be more than about 60 minutes, more than about 2 hours, more than about 5 hours, more than about 10 hours, more than about 1 day, more than about 2 days, more than about 3 days, or more than about 1 week apart. The optimal administration times will depend on the rates of metabolism, excretion, and/or the pharmacodynamic activity of the additional agent and the recombinant yeast cell being administered. Either the additional agent or the recombinant yeast cell may be administered first. For example, in one embodiment, the recombinant yeast cell is administered after administration of an antibiotic. In another embodiment, the recombinant yeast cell is administered after administration of a beta-lactamase.

[0108] Co-administration also does not require the therapeutic agents to be administered to the subject by the same route of administration. Rather, each therapeutic agent can be administered by any appropriate route, for example, parenterally or non-parenterally.

Methods of Treatment

[0109] In various aspects, the present invention provides methods for treating or preventing a *C. difficile* infection (CDI) and/or a *C. difficile*-associated disease, comprising administering an effective amount of a pharmaceutical composition (and/or additional agent) described herein to a subject or a patient need thereof.

[0110] In various embodiments, the CDI or *C. difficile*-associated disease is one or more of: *C. difficile* diarrhea (CDD), *C. difficile* intestinal inflammatory disease, colitis, pseudomembranous colitis, fever, abdominal pain, dehydration and disturbances in electrolytes, megacolon, peritonitis, and perforation and/or rupture of the colon.

[0111] In an embodiment, the present uses and methods pertain to co-treatment (simultaneously or sequentially) with the pharmaceutical compositions of the present invention and any additional agent described herein and/or any initial and/or adjunctive therapy. In another embodiment, the present uses and methods

pertain to treatment with a co-formulation of the pharmaceutical compositions of the present invention and any additional agent described herein and/or any initial and/or adjunctive therapy. In other embodiments, the present uses and methods pertain to treating a *C. difficile* infection (CDI) and/or a *C. difficile*-associated disease in a patient undergoing treatment with any additional agent described herein and/or any initial and/or adjunctive therapy described herein by administering a pharmaceutical composition of the present invention to the patient.

[0112] In various embodiments, the CDI and/or *C. difficile* associated disease is prevented by administration to a patient that is at risk for CDI and/or *C. difficile* associated disease (*e.g.* is undergoing or will undergoing antibiotic treatment, including IV antibiotic treatment and/or has previously been afflicted with CDI and/or *C. difficile* associated disease). In various embodiments, the CDI and/or *C. difficile* associated disease is treated or prevented in the context of initial onset or relapse/recurrence (*e.g.* due to continued or restarted antibiotic therapy). For example, in a patient that has previously suffered from CDI, the present pharmaceutical compositions (and/or additional agents) may be administered upon the first symptoms of recurrence. By way of non-limiting example, symptoms of recurrence include, in a mild case, about 5 to about 10 watery bowel movements per day, no significant fever, and only mild abdominal cramps while blood tests may show a mild rise in the white blood cell count up to about 15,000 (normal levels are up to about 10,000), and, in a severe case, more than about 10 watery stools per day, nausea, vomiting, high fever (*e.g.* about 102-104°F), rectal bleeding, severe abdominal pain (*e.g.* with tenderness), abdominal distention, and a high white blood count (*e.g.* of about 15,000 to about 40,000).

[0113] Regardless of initial onset or relapse/recurrence, CDI and/or *C. difficile* associated disease may be diagnosed via any of the symptoms described herein (*e.g.* watery diarrhea about 3 or more times a day for about 2 days or more, mild to bad cramping and pain in the belly, fever, blood or pus in the stool, nausea, dehydration, loss of appetite, loss of weight, *etc.*). Regardless of initial onset or relapse/recurrence, CDI and/or *C. difficile* associated disease may also be diagnosed via enzyme immunoassays *e.g.* to detect the *C. difficile* toxin A or B antigen and/or glutamine dehydrogenase (GDH), which is produced by *C. difficile* organisms), polymerase chain reaction (*e.g.* to detect the *C. difficile* toxin A or B gene or a portion thereof (*e.g. tcdA* or *tcdB*), including the ILLUMIGENE LAMP assay), a cell cytotoxicity assay. For example, any one of the following tests may be used may be used: Meridian Immuno*Card* Toxins A/B; Wampole Toxin A/B Quik Chek; Wampole *C. diff* Quik Chek Complete; Remel Xpect *Clostridium difficile* Toxin A/B; Meridian Premier Toxins A/B; Wampole *C. difficile* Tox A/B II; Remel Prospect Toxin A/B EIA; Biomerieux Vidas *C. difficile* Toxin A&B; BD Geneohm *C. diff*; Prodesse Progastro CD; and Cepheld Xpert *C. diff.* In various embodiments, the clinical sample is a patient stool sample. Also a flexible sigmoidoscopy "scope" test and/or an abdominal X-ray and/or a computerized tomography (CT) scan, which provides images of your colon, may be used in assessing a patient (*e.g.* looking for characteristic creamy white or yellow plaques adherent to the wall of the colon). Further, biopsies (*e.g.* of any region of the GI tract) may be used to assess a potential CDI and/or *C. difficile* associated disease patient.

[0114] Furthermore, the patients of the invention include, but are not limited to, patients that are at a particular risk for CDI and/or *C. difficile* associated disease, such as those which have been taking an antibiotic

during the past 30 or so days and/or have an immune system that is weak (*e.g.* from a chronic illness) and/or are women and/or are elderly (*e.g.* over about 65 years old) and/or are elderly woman and/or undergo treatment with for heartburn or stomach acid disorders (*e.g.* with agents such as PREVACID, TAGAMET, PRILOSEC, or NEXIUM and related drugs) and/or have recently been in the hospital, including in an intensive care unit, or live in a nursing home. Accordingly, in some embodiments, the pharmaceutical composition of the present invention may be used to prophylactically prevent CDI and/or *C. difficile* associated disease.

[0115] In some embodiments, the methods and uses of the present invention relate to a patient is undergoing treatment or has recently undergone treatment with one or more primary antibiotic. A "primary antibiotic" refers to an antibiotic that is administered to a patient and which may result in CDI and/or *C. difficile* associated disease. These include the antibiotics that most often lead to CDI and/or *C. difficile* associated disease, such as, for example, fluoroquinolones, cephalosporins, clindamycin and penicillins.

[0116] In some embodiments, the methods and uses of the present invention include those in which an initial and/or adjunctive therapy is administered to a patient. Initial and/or adjunctive therapy indicates therapy that is used to treat CDI and/or *C. difficile* associated disease upon detection of such disease. In some embodiments, the initial and/or adjunctive therapy is one or more of metronidazole, vancomycin, fidaxomicin, rifaximin, fecal bacteriotherapy, probiotic therapy, and antibody therapy, as described herein. In various embodiments, the methods and uses of the present invention include use of the inventive pharmaceutical composition as an adjuvant to any of these initial and/or adjunctive therapies (including co-administration or sequential administration). In various embodiments, the methods and uses of the present invention include use of the inventive pharmaceutical composition in a patient undergoing initial and/or adjunctive therapies.

[0117] In some embodiments, the terms "patient" and "subject" are used interchangeably. In some embodiments, the subject and/or animal is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In other embodiments, the subject and/or animal is a non-mammal, such, for example, a zebrafish. In some embodiments, the subject and/or animal may comprise fluorescently-tagged cells (with *e.g.* GFP). In some embodiments, the subject and/or animal is a transgenic animal comprising a fluorescent cell.

[0118] In some embodiments, the subject and/or animal is a human. In some embodiments, the human is a pediatric human. In other embodiments, the human is an adult human. In other embodiments, the human is a geriatric human. In other embodiments, the human may be referred to as a patient.

[0119] In certain embodiments, the human has an age in a range of from about 6 to about 18 months old, from about 18 to about 36 months old, from about 1 to about 5 years old, from about 5 to about 10 years old, from about 10 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old,

from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old, from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old.

[0120] In other embodiments, the subject is a non-human animal, and therefore the invention pertains to veterinary use. In a specific embodiment, the non-human animal is a household pet. In another specific embodiment, the non-human animal is a livestock animal.

Kits

[0121] The present invention is also directed to a kit for the treatment of *C. difficile* infections and *C. difficile*-associated diseases. The kit is an assemblage of materials or components, including at least one of the inventive pharmaceutical compositions described herein. Thus, in some embodiments, the kit contains at least one of the pharmaceutical compositions described herein.

[0122] The exact nature of the components configured in the kit depends on its intended purpose. In one embodiment, the kit is configured for the purpose of treating human subjects.

[0123] Instructions for use may be included in the kit. Instructions for use typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as to treat a *C. difficile* infection or a *C. difficile*-associated disease. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

[0124] The materials and components assembled in the kit can be provided to the practitioner store in any convenience and suitable ways that preserve their operability and utility. For example, the components can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging materials. In various embodiments, the packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. The packaging material may have an external label which indicates the contents and/or purpose of the kit and/or its components.

Definitions

[0125] As used herein, “a,” “an,” or “the” can mean one or more than one.

[0126] Further, the term “about” when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 10% of that referenced numeric indication. For example, the language “about 50” covers the range of 45 to 55.

[0127] An “effective amount,” when used in connection with medical uses is an amount that is effective for providing a measurable treatment, prevention, or reduction in the rate of pathogenesis of a disease of interest.

[0128] As used herein, something is "decreased" if a read-out of activity and/or effect is reduced by a significant amount, such as by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100%, in the presence of an agent or stimulus relative to the absence of such modulation. As will be understood by one of ordinary skill in the art, in some embodiments, activity is decreased and some downstream read-outs will decrease but others can increase.

[0129] Conversely, activity is "increased" if a read-out of activity and/or effect is increased by a significant amount, for example by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100% or more, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 50-fold, at least about 100-fold, in the presence of an agent or stimulus, relative to the absence of such agent or stimulus.

[0130] As referred to herein, all compositional percentages are by weight of the total composition, unless otherwise specified. As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the compositions and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features.

[0131] Although the open-ended term "comprising," as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as "consisting of" or "consisting essentially of."

[0132] As used herein, the words "preferred" and "preferably" refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the technology.

[0133] The amount of compositions described herein needed for achieving a therapeutic effect may be determined empirically in accordance with conventional procedures for the particular purpose. Generally, for administering therapeutic agents for therapeutic purposes, the therapeutic agents are given at a pharmacologically effective dose. A "pharmacologically effective amount," "pharmacologically effective dose," "therapeutically effective amount," or "effective amount" refers to an amount sufficient to produce the desired physiological effect or amount capable of achieving the desired result, particularly for treating the disorder or disease. An effective amount as used herein would include an amount sufficient to, for example, delay the

development of a symptom of the disorder or disease, alter the course of a symptom of the disorder or disease (*e.g.*, slow the progression of a symptom of the disease), reduce or eliminate one or more symptoms or manifestations of the disorder or disease, and reverse a symptom of a disorder or disease. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

[0134] Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to about 50% of the population) and the ED50 (the dose therapeutically effective in about 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. In some embodiments, compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from in vitro assays, including, for example, cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 as determined in cell culture, or in an appropriate animal model. Levels of the described compositions in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

[0135] In certain embodiments, the effect will result in a quantifiable change of at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, or at least about 90%. In some embodiments, the effect will result in a quantifiable change of about 10%, about 20%, about 30%, about 50%, about 70%, or even about 90% or more. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

[0136] As used herein, "methods of treatment" are equally applicable to use of a composition for treating the diseases or disorders described herein and/or compositions for use and/or uses in the manufacture of a medicaments for treating the diseases or disorders described herein.

[0137] This invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Construction of Heteromultimeric Binding Proteins Against *C. Difficile* Toxins

[0138] The anti-toxin A VHHs AH3 and AA6 and the anti-Toxin B VHHs 5D and E3 were constructed as heterodimers. Sequences of AH3, AA6, 5D, and E3 are depicted in **Figure 1**.

[0139] A total of eight gene constructs were designed, synthesized, and evaluated, two anti-Toxin A, and six anti-Toxin B gene expression constructs:

1. Toxin A: AH3 + AA6 wildtype (wt) (using the sequences with the protease-resistant linker sequence GGGGSGGGGSGGGGS (SEQ ID NO: 22).

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCAASGFTLDYSSIGWFRQA
PGKEREGVSCISSSGDSTKYADSVKGRFTTSRDNAKNTVYLQMNSLKPDD
TAVYYCAAFRATMCGVFPLSPYGKDDWGKGTLVTVSSEPKTPKPQ**GGGG**
SGGGGSGGGGSQGVQSQVQLVESGGGLVQPGGSLRLSCAASGFTFSDY
VMTWVRQAPGKGPEWIATINTDGSTMRDDSTKGRFTISRDNAKNTLYLQM
TSLKPEDTALYYCARGRVISASAIRGAVRGPGTQVTVSSEPKTPKPQGGGA
R**GAPVPYPDPLEPRHHHHHH** (SEQ ID NO:41)

DNA Sequence

ATGGCGGCCGCTCAAGGAGTTCAATCACAATTACAATTAGTCGAAAGTG
GAGGAGGGTTAGTTCAGCCTGGAGGTTCCCTAAGATTATCTTGCGCAG
CTTCAGGTTTCACATTGGATTACTCTAGCATCGGCTGGTTCAGACAAGC
TCCTGGTAAGGAAAGAGAAGGTGTGTCCTGTATTTCTTCAAGTGGAGAC
AGCACAAAATACGCTGACTCAGTCAAGGGCCGTTTCACTACATCAAGAG
ACAACGCCAAAAACACAGTTTACTTGCAGATGAACTCTTTGAAGCCAGA
TGATACCGCAGTATACTATTGTGCAGCTTTTAGAGCCACAATGTGCGGT
GTGTTTCCACTGTCCCCATACGGTAAGGATGATTGGGGAAAGGGTACA
TTGGTTACAGTGTCCTCTGAACCAAAAACTCCTAAACCACAGGGGGGTG
GTGGTTCTGGTGGTGGTGGCTCCGGTGGGGGTGGCTCACAGGGTGTT
CAATCTCAAGTACAATTGGTAGAATCAGGGGGTGGTCTTGTGCAACCAG
GTGGATCACTAAGACTATCTTGTGCCGCTTCTGGATTCACTTTCTCTGA
CTACGTCATGACATGGGTTAGACAAGCACCAGGAAAGGGCCCAGAGTG
GATTGCAACTATCAATACCGATGGTAGTACTATGAGAGATGACTCTACA
AAGGGCAGATTTACAATATCTCGTGATAACGCTAAAAATACTTTGTACCT
CCAAATGACCTCACTTAAGCCAGAGGATACTGCACTGTATTACTGCGCT
AGAGGCAGAGTTATCAGTGCATCTGCAATTAGAGGTGCTGTCAGAGGC
CCAGGTACCCAAGTTACTGTTTCATCAGAACCTAAGACACCAAAACCTC
AGGGGGGGGGTGCCAGAGGCGCGCCAGTCCCTTACCCAGATCCACTT
GAGCCTAGACATCATCACCATCATCACTAG (SEQ ID NO:42)

2. Toxin A: AH3 + AA6 with J4 region substitution. Using llama VHHs it was demonstrated that secretion efficiency could be improved >100-fold in S. *cerevisiae* by using the J4 region compared to the J7 region. The J region (encoded by 7 alpaca J genes transverses the CDR3 and the FR4 region of VHHs (**Figure 2**). Therefore the FR4 J region of the AH3 and AA6 VHHs were replaced with the alpaca J4 region. (*See* **Figures 2** and **3**).

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCAASGFTLDYSSIGWFRQA
PGKEREGVSCISSSGDSTKYADSVKGRFTTSRDNAKNTVYLQMNSLKPDD
TAVYYCAAFRATMCGVFPLSPYGKDDWG**Q**GT**Q**VTVSSEPKTPKPQ**GGG**
GSGGGGSGGGGSQGVQSQVQLVESGGGLVQPGGSLRLSCAASGFTFSD
YVMTWVRQAPGKGPEWIATINTDGSTMRDDSTKGRFTISRDNAKNTLYLQ
MTSLKPEDTALYYCARGRVISASAIRGAV**W**G**Q**GTQVTVSSEPKTPKPQGG
GAR**GAPVPYPDPLEPRHHHHHH** (SEQ ID NO:43)

DNA Sequence

ATGGCGGCCGCTCAAGGAGTTCAATCACAATTACAATTAGTCGAAAGTG
GAGGAGGGTTAGTTCAGCCTGGAGGTTCCCTAAGATTATCTTGCGCAG
CTTCAGGTTTCACATTGGATTACTCTAGCATCGGCTGGTTCAGACAAGC
TCCTGGTAAGGAAAGAGAAGGTGTGTCCTGTATTTCTTCAAGTGGAGAC
AGCACAAAATACGCTGACTCAGTCAAGGGCCGTTTCACTACATCAAGAG
ACAACGCCAAAAACACAGTTTACTTGCAGATGAACTCTTTGAAGCCAGA
TGATACCGCAGTATACTATTGTGCAGCTTTTAGAGCCACAATGTGCGGT
GTGTTTCCACTGTCCCCATACGGTAAGGATGATTGGGGACAAGGTACA
CAAGTTACAGTGTCCTCTGAACCAAAAACTCCTAAACCACAGGGGGGT
GGTGGTTCTGGTGGTGGTGGCTCCGGTGGGGGTGGCTCACAGGGTGT
TCAATCTCAAGTACAATTGGTAGAATCAGGGGGTGGTCTTGTGCAACCA
GGTGGATCACTAAGACTATCTTGTGCCGCTTCTGGATTCACTTTCTCTG
ACTACGTCATGACATGGGTTAGACAAGCACCAGGAAAGGGCCCAGAGT
GGATTGCAACTATCAATACCGATGGTAGTACTATGAGAGATGACTCTAC
AAAGGGCAGATTTACAATATCTCGTGATAACGCTAAAAATACTTTGTACC
TCCAAATGACCTCACTTAAGCCAGAGGATACTGCACTGTATTACTGCGC
TAGAGGCAGAGTTATCAGTGCATCTGCAATTAGAGGTGCTGTCTGGGG
CCAGGGTACCCAAGTTACTGTTTCATCAGAACCTAAGACACCAAAACCT
CAGGGGGGGGGTGCCAGAGGCGCGCCAGTCCCTTACCCAGATCCACT
TGAGCCTAGACATCATCACCATCATCACTAG (SEQ ID NO:44)

3. Toxin B: 5D + E3 wild type (wt, using the sequences with the protease-resistant linker sequence GGGGSGGGGSGGGGS (SEQ ID NO: 22).

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKREDTA
VYYCARRRFSASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET

VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELRGQGIQVTVSSEPKTPKPQGGGARGAPVPYPDP
LEPRHHHHHH (SEQ ID NO:45)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG
AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGAGA
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGAAGAGGTAC
ACAAGTTGCAGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG
TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG
TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTAAGAGGTCAAGGTATTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:46)

4. Toxin B: E3 + 5D with the alpaca J4 region. The FR4 J region of the E3 and 5D VHHs was replaced with the alpaca J4 region. (**Figures 2** and **3**).

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKREDTA
VYYCARRRFSASSVNRWLADDYDVWGQGTQVTVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET
VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELWGQGTQVTVSSEPKTPKPQGGGARGAPVPYPD
PLEPRHHHHHH (SEQ ID NO:47)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG
AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGAGA
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGACAAGGTAC
ACAAGTTACCGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG
TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG
TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTATGGGGTCAAGGTACTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:48)

5. Toxin B: 5D + E3 with the KR sequence in 5D FR3 (QMNSL**KR**EDTAVYY (SEQ ID NO:53) changed to a KP sequence. The endogenous yeast protease Kex2 cleaves most efficiently at K/R but cleavage can also occur at R/R sites. Changing the 5D KR to KP would block cleavage by Kex2. However, the CDR3 region of 5D has an RRR sequence that potentially can be cleaved by Kex2. Kex2 cleaves proteins in the Golgi, therefore it is possible that the VHH will be already folded and the RRR sequence may not be available for cleavage. The RRR sequence is not changed as it is part of the antigen recognition site. (**Figure 1**).

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKPEDTA
VYYCARRRFSASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET
VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELRGQGIQVTVSSEPKTPKPQGGGARGAPVPYPDP
LEPRHHHHHH (SEQ ID NO:49)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG
AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGCCT
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGAAGAGGTAC
ACAAGTTGCAGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG
TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG
TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTAAGAGGTCAAGGTATTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:50)

6. Toxin B: E3 + 5D with the KP change with the J4 region.

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKPEDTA
VYYCARRRFSASSVNRWLADDYDVWGQGTQVTVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET
VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELWGQGTQVTVSSEPKTPKPQGGGARGAPVPYPD
PLEPRHHHHHH (SEQ ID NO:51)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG

AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGCCT
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGACAAGGTAC
ACAAGTTACCGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG
TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG
TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTATGGGGTCAAGGTACTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:52)

7. Toxin B: E3 + 5D KA mutant. The KR sequence in 5D FR3 (QMNSL**KR**EDTAVYY (SEQ ID NO:53) changed to a KA. The use of an A substitution is generally considered to be a benign change.

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKAEDTA
VYYCARRRFSASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET
VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELRGQGIQVTVSSEPKTPKPQGGGARGAPVPYPDP
LEPRHHHHHH (SEQ ID NO:54)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG
AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGGCA
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGAAGAGGTAC
ACAAGTTGCAGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG

TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG
TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTAAGAGGTCAAGGTATTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:55)

8. Toxin B: E3 + 5D KK mutant. The KR sequence in 5D FR3 (QMNSL**KR**EDTAVYY (SEQ ID NO:53) changed to a KK. K is the most similar amino acid to R.

Amino Acid Sequence

MAAAQGVQSQLQLVESGGGLVQPGGSLRLSCEASGFTLDYYGIGWFRQP
PGKEREAVSYISASARTILYADSVKGRFTISRDNAKNAVYLQMNSLKKEDTA
VYYCARRRFSASSVNRWLADDYDVWGRGTQVAVSSEPKTPKPQGGGGS
GGGGSGGGGSQGVQSQVQLVESGGGLVQTGGSLRLSCASSGSIAGFET
VTWSRQAPGKSLQWVASMTKTNNEIYSDSVKGRFIISRDNAKNTVYLQMN
PLKPEDTGVYFCKGPELRGQGIQVTVSSEPKTPKPQGGGARGAPVPYPDP
LEPRHHHHHH (SEQ ID NO:56)

DNA Sequence

ATGGCGGCCGCACAAGGAGTTCAATCACAATTACAGTTAGTCGAGTCA
GGTGGAGGATTGGTACAACCAGGAGGTAGCTTAAGATTGAGCTGCGAA
GCATCAGGTTTCACACTTGATTACTACGGTATCGGTTGGTTTCGTCAAC
CACCAGGGAAGGAACGTGAAGCAGTGTCCTACATATCAGCTTCTGCCA
GAACTATCTTGTACGCTGATTCTGTGAAGGGCAGATTCACCATTTCTAG
AGATAATGCTAAAAACGCTGTCTACCTACAAATGAATTCCTTGAAGAAG
GAAGATACTGCCGTTTACTACTGTGCTAGACGTAGATTCTCTGCTTCCT
CTGTTAATAGATGGCTAGCCGATGACTATGATGTATGGGGAAGAGGTAC
ACAAGTTGCAGTATCCTCAGAGCCTAAGACACCAAAGCCACAAGGAGG
CGGGGGAAGTGGTGGGGGTGGTTCAGGTGGTGGTGGTTCTCAGGGCG
TGCAAAGTCAAGTCCAGCTTGTAGAATCTGGGGGCGGGCTGGTTCAGA
CTGGTGGATCACTGAGACTTAGTTGTGCCTCCTCTGGCTCTATTGCTGG
TTTTGAAACCGTTACATGGTCTAGACAAGCACCAGGCAAATCATTACAG
TGGGTCGCATCAATGACTAAGACAAACAACGAAATCTACAGTGACTCTG

TTAAGGGTAGATTTATCATTTCAAGAGACAACGCTAAAAACACTGTGTAC
TTGCAAATGAACCCTTTGAAACCAGAGGATACAGGCGTCTACTTCTGCA
AGGGTCCAGAACTAAGAGGTCAAGGTATTCAAGTTACAGTTTCTTCTGA
ACCTAAAACCCCTAAGCCTCAGGGTGGTGGAGCAAGAGGCGCGCCAG
TTCCATATCCAGATCCACTCGAGCCTAGACATCATCATCACCACCATTA
G (SEQ ID NO:57)

[0140] All constructs were codon optimized for S. *cerevisiae*, modified to reduce DNA homologies (there are several regions of conserved sequence within the VHH) (**Figure 1**), checked for N-linked glycosylation sites, synthesized and cloned into the yeast expression plasmid, pD1214 (DNA2.0 Menlo Park, CA), that contains the strong, constitutive TEF promoter. Different *S. cerevisiae* secretion vectors containing a panel of different leader sequences to facilitate secretion are known. Importantly, llama VHHs were expressed most efficiently in S. *cerevisiae* using the yeast invertase signal. An epitope tag (E-tag) and a 6-His-tag was included on the COOH end of the VHH heterodimers to facilitate visualization by Western and protein purification if desired.

Example 2: Yeast Expression of Heteromultimeric Binding Proteins Against *C. Difficile* Toxins

[0141] All constructs as described above were synthesized and cloned into the yeast expression vector pD1214. A series of *S. cerevisiae* secretion vectors are available which contain a panel of different leader sequences to facilitate secretion (DNA2.0 Menlo Park, CA). An illustrative secretion signal is the yeast mating factor alpha (MAT alpha) signal, which is an 89 amino acid sequence composed of the signal and the prosequence which is cleaved in the Golgi by Kex2, an endogenous yeast protease, to yield the mature, secreted protein. The invertase and other signal sequences are naturally cleaved during translocation and secretion of the protein by signal peptidase and do not require additional protease cleavage steps. Llama VHHs can be efficiently expressed in *S. cerevisiae* using the yeast invertase signal. Illustrative signal sequences are described in Table 1 below:

Table 1: Signal Sequences

Abbrev.	Name	Sequence	Length
FAKS	Alpha-factor_full (*S.cerevisiae*)	*MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIG YSDLEGDFDVAVLPFSNSTNNGLLFINTIASIAAKEEGVSL EKREAEA (SEQ ID NO:58)	89 aa
AKS	Alpha-factor_kex_ste (*S.cerevisiae*)	*MRFPSIFTAVLFAASSALAAPVNTTTEDELEGDFDVAVL PFSASIAAKEEGVSLEKREAEA (SEQ ID NO:59)	42 aa
AK	Alpha-factor_kex (*S. cerevisiae*)	*MRFPSIFTAVLFAASSALAAPVNTTTEDELEGDFDVAVL PFSASIAAKEEGVSLEKR (SEQ ID NO:60)	38 aa
AT	Alpha-factor_T (*S. cerevisiae*)	*MRFPSIFTAVLFAASSALA (SEQ ID NO:61)	19 aa
AA	Alpha-amylase (*Aspergillus niger*)	MVAWWSLFLYGLQVAAPALA (SEQ ID NO:62)	20 aa
GA	Glucoamylase (*Aspergillus awamori*)	MSFRSLLALSGLVCSGLA (SEQ ID NO:63)	18 aa
IN	Inulinase (*Kluyveromyces*	MKLAYSLLLPLAGVSA (SEQ ID NO:64)	16 aa

	maxianus)		
IV	Invertase (*S.cerevisiae*)	MLLQAFLFLLAGFAAKISA (SEQ ID NO:65)	19 aa
KP	Killer protein (*S.cerevisiae*)	MTKPTQVLVRSVSILFFITLLHLVVA (SEQ ID NO:66)	26 aa
LZ	Lysozyme (*Gallus gallus*)	MLGKNDPMCLVLVLLGLTALLGICQG (SEQ ID NO:67)	26 aa
SA	Serum albumin (*Homo sapien)*	MKWVTFISLLFLFSSAYS (SEQ ID NO:68)	18 aa

[0142] Three signal sequences were tested: Invertase, FAKS (Alpha-factor full), and AT (Alpha-factor). A total of 6 plasmids against toxin A (AA) and 17 plasmids against toxin B (BB) were generated.

Table 2. AA Yeast Expression Plasmids

Plasmid Number	Gene	Signal Sequence
+ control	cutinase	
179081	AH3 + AA6	AT (short)
179082	AH3 + AA6 J4	AT
179083	AH3 + AA6	FAKS (long)
179084	AH3 + AA6 J4	FAKS
179085	AH3 + AA6	Invertase
179086	AH3 + AA6 J4	Invertase

Table 3. BB Yeast Expression Plasmids

Plasmid #	Gene	Signal Sequence
+ control	cutinase	
153270	5D + E3 wt	Invertase
153271	5D + E3 KP mutant	Invertase
153272	5D + E3 wt + J4	Invertase
153273	5D + E3 KP + J4	Invertase
153274	5D + E3 KK mutant	Invertase
153275	5D + E3 KA mutant	Invertase
153276	5D + E3 wt	FAKS (long)
153277	5D + E3 KP mutant	FAKS
153278	5D + E3 wt + J4	FAKS
153279	5D + E3 KP + J4	FAKS
153280	5D + E3 KK mutant	FAKS
153281	5D + E3 KA mutant	FAKS
153282	5D + E3 wt	AT (short)
153283	5D + E3 KP mutant	AT
153284	5D + E3 wt + J4	AT
153285	5D + E3 KP + J4	AT
153286	5D + E3 KK mutant	AT
153287	5D + E3 KA mutant	AT

[0143] All 6 AA plasmids, and 10 BB plasmids (153270, 153271, 153272, 153273, 153274, 153275, 153276, 153279, 153280, 153281) were transformed into *S. cerevisiae* strain INVSc 1 (Invitrogen, Cat # C810-00), which is a URA3- strain. The cells were made competent and transformed cells were plated to CM-URA agar (Teknova C3080) and incubated at 30°C for 3 days. The positive control vector, 120955 (pD1214-Invertase-Cutinase, 22 kDa) was also transformed. Two colonies from each plate were picked into 2 ml CM-URA broth (Tecknova C8140) in a 24-well, deep-well plate. As negative controls, two colonies of strain INVSc 1 were picked into CM-URA+50 ug/ml uracil broth. Plates were covered with a breathable seal and incubated for 2 days at 250 rpm at 30°C. One ml of each culture was transferred to 4 ml YPD broth (Teknova Y5000) in a 24-well plate. Plates were covered with a breathable seal and incubated for 2 days at 250 rpm and 30°C. Plates were centrifuged for 5 minutes at 500x g. Three ml of each supernatant was removed to a 15-ml screw-capped tube. Sixty µl of supernatants were mixed with 20 µl of 4X samples buffer and heated for 10 min at 95°C then cooled to 12°C. Fifteen µl of samples were run on a 4-12% Novex SDA/PAGE gel with MES buffer and Sea Blue Plus 2 markers. Gels were stained with SimplyBlue SafeStain. Supernatants were flash frozen and stored at -80°C.

[0144] For Western blot, 17.5 µl of samples were loaded on a 4-12% Novel SDS/PAGE gel with MES buffer and SeaBlue Plus 2 markers. After running, gels were blotted to PVDF membranes. The membranes were blocked with Pierce PBS Superblock for 15 min, then incubated with rocking overnight with a 1:1000 dilution of Qiagen Mouse Tetra-His Antibody in PBST buffer + 10% Superblock. Following 3 washes in PBST buffer, Goat Anti-Mouse IgG-HRP was added to the blot at 1:10,000 dilution in PBST buffer + 10% Superblock. The blot was incubated for 1 hour with secondary antibody, washed 3 times in PBST buffer. The blot was developed with Peirce 1-Step TMB-Blotting Substrate Solution and stopped by washing with two changes of water.

[0145] For protein quantitation, Pierce Bovine Serum Albumin (BSA) protein standard was diluted to 300, 100, 33.3, 11.1, and 3.7 µg/ml and run along with samples of culture supernatants as described. The gel was scanned and imported into TotalLab100, where relevant bands were selected and quantitated by densitometry. A standard curve generated from the BSA bands was used to determine concentrations of the cutinase control and the ~38 kDa band from the test samples.

[0146] For both the AAs (**Figure 4**) and the BBs (**Figure 6**), a specific protein band of ~38 kDa was observed. The AAs were expected to be 33.1 kDa and the BBs to be 32.7 kDa, therefore the proteins appear to be running larger than expected on the SDS/PAGE. To verify that the 38 kDa bands were indeed the expressed VHH proteins, Western analyses were performed using an antibody specific to the 6-His tag (**Figures 5** and **7**). The Western demonstrated that the 38 kDa bands were the expressed VHH proteins.

[0147] There appeared to be much variation in expression levels between the two transformants from each plasmid evaluated, as well as between different plasmids. The highest expressing clones were chosen for a rough protein quantitation evaluation by SDS/PAGE analysis with a BSA-derived standard curve with densitometry scanning quantitation (**Figure 8**). The results of the quantitation are shown in **Tables 5A and 5B**.

Table 5A. Yeast Expression: AA Protein Quantitation

Plasmid Number	Gene	Signal Sequence	Protein Expression* (ug/ml)
+ control	cutinase	Invertase	6.25
179081	AH3 + AA6	AT (short)	11.46
179082	AH3 + AA6 J4	AT	11.1
179083	AH3 + AA6	FAKS (long)	6.99
179084	AH3 + AA6 J4	FAKS	7.26
179085	AH3 + AA6	Invertase	0.90
179086	AH3 + AA6 J4	Invertase	13.67

*based on higher expressing clone

Table 5B. Yeast Expression: BB Protein Quantitation

Plasmid #	Gene	Signal Sequence	Protein Exp* (ug/ml)
+ control	cutinase	Invertase	6.25
153270	5D + E3 wt	Invertase	6.33
153271	5D + E3 KP mutant	Invertase	0.44
153272	5D + E3 wt + J4	Invertase	5.23
153273	5D + E3 KP + J4	Invertase	41.32
153274	5D + E3 KK mutant	Invertase	3.34
153275	5D + E3 KA mutant	Invertase	3.30
153276	5D + E3 wt	FAKS (long)	0.65
153277	5D + E3 KP mutant	FAKS	ND
153278	5D + E3 wt + J4	FAKS	ND
153279	5D + E3 KP + J4	FAKS	7.20
153280	5D + E3 KK mutant	FAKS	3.72
153281	5D + E3 KA mutant	FAKS	0.42
153282	5D + E3 wt	AT (short)	ND
153283	5D + E3 KP mutant	AT	ND
153284	5D + E3 wt + J4	AT	ND
153285	5D + E3 KP + J4	AT	ND
153286	5D + E3 KK mutant	AT	ND
153287	5D + E3 KA mutant	AT	ND

*Based on higher expressing clone, ND is not done.

[0148] The expression levels were variable but based on the quantitation analysis the highest AA expresser were the AH3 + AA6 with the J4 region substitution, with any of the signals, with the AT short being

the best. The next highest expressers were the AH3+AA6 wild type with the AT signal. For the BB proteins, the highest expressers were again the clones with the J4 region substitution, and wild type and the KK mutant.

Example 3. Activity Analysis of Yeast Expressed VHHs

[0149] Cell-based neutralization assays against *C. difficile* Toxin A or Toxin B were performed. In these assays, Vero cells, were exposed to *C. difficile* Toxin A or Toxin B in the presence of 5-fold dilutions of the yeast supernatants (ranging from 10 µl to 0.00013 µl) containing the anti-Toxin A or Toxin B VHH heterodimers and incubated for 24 hours. The percentage of cell rounding was monitored using a phase contrast microscope. The supernatant from the cutinase control (120955) was used as the negative control. The 0.2 um filtered yeast supernatants were used as is, and the amount of VHH protein in the supernatants was not normalized based on the protein quantitation gel. Thus, the strongest (or most concentrated) VHH produced the greatest protection at the highest dilution.

[0150] Neutralization assays were performed for *C. difficile* Toxin A and Toxin B. For both assays, toxin kill curves were performed to demonstrate assay robustness and to verify the use of 50 pM of Toxin A or 40 fM of Toxin B as the challenge for the neutralization assays. (**Figure 9**). For the anti-Toxin A VHHs, all six yeast supernatants neutralized Toxin A **(Figure 10**). The potencies of the yeast supernatants expressing the VHHs with the J4 modifications appeared weaker than the wild type VHHs. Importantly, the VHHs with the J4 modifications displayed the highest protein concentrations, suggesting that their potencies were significantly reduced. The best AA candidates appear to be 179081 (wt-AT) and 179083 (wt-FAKS).

[0151] For the anti-Toxin B VHHs, all ten yeast supernatants clearly neutralized Toxin B (**Figure 11**). The potencies of the VHHs with the J4 modifications were clearly weaker than the wild type or KR substitution mutants (*i.e.*, KP, KK, KA) although the J4 mutants displayed higher protein expression. Surprisingly, 153280 (KK mutant with the FAKS signal) showed approx. 5-fold higher potency than the other non-J4 VHHs. Very good BB candidates appear to be 153275 (KA mutant with Invertase signal) and 153280 (KK with FAKS signal).

[0152] Accordingly the yeast S. *cerevisiae* is able to secrete biologically active *C. difficile* anti-toxin A and B VHHs. All VHH heterodimer expression cassettes mediated the expression of biologically active proteins. The J4 region modification did improve protein secretion from the yeast; however, these changes resulted in reduced activity in the *C. difficile* toxin neutralization assays. Unexpectedly, the KK change in the anti-Toxin B 5D VHH resulted in 5-fold higher activity levels (**Figure 11**). Additionally, the KA and KP mutants all showed comparable if not better biological activity than the wild type 5D VHH, which was also an unexpected result, since in most cases, the 5D region mutations resulted in lower protein expression levels compared to wild type.

[0153] Four constructs are chosen to generate stably transformed *S. boulardii* strains for orally delivering the VHH-expressing yeast to treat and prevent *C. difficile* infection in animal disease models (for example, hamster, mouse, and pig models of *C. difficile* infection).

Example 4. Generation of S. *cerevisiae*, Substrain *boulardii,* Transformants that Secrete *C. difficile* Anti-Toxin A and/or Anti-Toxin B VHHs.

[0154] S. *cerevisiae*, substrain *boulardii* (S. *boulardii*) is a wild-type yeast strain that has been marketed for over 40 years as a probiotic. It has been used for the prevention and the treatment of diarrheal diseases, including antibiotic-associated diarrhea and *C. difficile* infection (reviewed by Kelesidis and Pothoulakis, 2012; Hatoum *et al.*, 2012). S. *boulardii* differs from other S. *cerevisiae* strains as the optimal growth temperature of S. *boulardii* is 37°C while other strains prefer lower temperatures (between 30 and 33°C), S. *boulardii* is resistant to low pH and is highly tolerant to bile acids (Edwards-Ingram *et al.*, 2007; Graff *et al.*, 2008). S. *boulardii* was demonstrated to survive the intestinal tract in humans (Klein *et al.*, 1993) where 0.1% viable yeast was recovered in feces after a single administration of 10^{10} cells. Concurrent antibiotic treatment increased recovery two-fold (Klein *et al.*, 1993). S. *boulardii* is used as a vehicle for delivery of the *C. difficile* Anti-Toxin A and Anti-Toxin B VHHs directly to the digestive tract.

[0155] Two strategies are used to genetically modify S. *boulardii*. One is the production of a S. *boulardii* URA3 knockout strain to allow the use of the VHH AA plasmids (Table 2) and VHH BB plasmids (Table 3) that contain the URA3 selectable marker to generate transformants to use in efficacy evaluation in rodents and/or pigs. The S. *boulardii* URA3 knockout is generated using the CRISPR recombination system (DiCarlo et al, 2013, Nucleic Acids Res. 41:4436). The S. *boulardii* strain, designation Sb48 (ATCC Product # MYA-796) submitted to ATCC by D. A. Stevens (McCullough *et al.*, 1998; J. Clinical Microbiology, 36:2613) is used for these studies. Three potential wild-type Cas9 cleavage sites in the upstream region of the URA3 gene are identified and approximately 500 pb of the regions surrounding these target sites are sequenced to ensure the presence of the sites in this yeast strain. A homology construct is designed that contains an approximate 10 bp region in the middle replaced by an insert that contains multiple stop codons in all frames ensuring that the first stop codon is in the URA3 reading frame. The CRISPR system is used to create the recombination/insertion and the URA3- clones are selected on FOA (5-fluoroorotic acid) media. 5-FOA allows the selection for URA3- mutants, as an active URA3 gene (encodes orotidine 5'-phosphate decarboxylase) converts FOA into a toxic compound causing cell death. The selected clones are then tested to ensure that they will not grow on media without uracil. Selected clones are sequenced to verify the expected integration. Once the S. *boulardii* strain is confirmed to be URA3-, the yeast are transformed with the VHH Toxin A (Table 2) and VHH Toxin B (Table 3) plasmids. Clones are identified by plating on media without uracil. The resulting transformants are screened for secretion of the 38 kDa protein using SDS/PAGE (*see* **Figures 4** and **6**) and the identity of the proteins are verified by Western analyses (*see* **Figures 5** and **7**). Filtered yeast supernatants are evaluated for activity using the cell-based toxin neutralization assay (*see* **Figures 10** and **11**). A similar strategy is used to genetically modify *Pichia pastoris* to generate transformants that express the VHH Toxin A (Table 2) and VHH Toxin B (Table 3) plasmids.

[0156] The second strategy generates stable integrants in the wild-type S. *boulardii* strain using a neomycin resistance gene (neo) as the selectable marker. Without neo expression, S. *boulardii* is sensitive to G418. The S. *boulardii* strain, designation Sb48 (ATCC Product # MYA-796) submitted to ATCC by D. A.

Stevens (McCullough *et al.*, 1998; J. Clinical Microbiology, 36:2613) is used for these studies. Integration regions are chosen based on Flagfeldt et al (2009, Yeast 26:545), where chromosomal integration sites were screened for high level heterologous gene expression. The integration sites that show the highest expression levels, Regions 20, 21, and 19 are sequenced in the wild-type S. *boulardii* strain to verify their presence. Once verified, a region is chosen and plasmids containing integration cassettes are designed. The integration cassettes contain a VHH expression sequence chosen from Table 1 and/or Table 2, a neo expression cassette, at least 500 bp of homology sequence from the upstream part of the integration region and at least 500 bp of homology sequence from the downstream part of the integration region so that the integration region is deleted via the homologous recombination event. The wild-type S. *boulardii* is transformed with the integration cassettes and clones are selected for G418 resistance. Clones are picked, cultures grown, and supernatants screened for the presence of the 38 kDa protein via SDS/PAGE (*see* Figures 4 and 6). Filtered yeast supernatants are evaluated for activity using the cell-based toxin neutralization assay (*see* **Figures 10** and **11**). Excellent clones, based on protein expression levels and biological activity, are chosen and the insert is sequenced to verify the integrity of the integrated sequence. A similar strategy is utilized to generate stable inegrants in *Pichia pastoris*.

Example 5: *In Vivo* Analysis of Yeast Expressed VHHs in Rodent Models of *C. difficile* Disease

[0157] The S. *boulardii* transformants expressing the *C. difficile* anti-Toxin A or anti-Toxin B VHHs are evaluated in rodent models of *C. difficile* disease (CDI), including the Syrian Golden hamster (*Mesocricetus auratus*) *C. difficile* model (Sambol and Tang, 2001; J. Infect. Disease 183:1760). The hamster model has been referred to as "the gold standard" small animal model for the evaluation of the efficacy of a variety of prophylactic and therapeutic interventions against CDI. CDI is induced in the hamsters using the following protocol. Male Golden Syrian hamsters, purchased from Harlan (Indianapolis, IN) are pretreated 5 days or 24 hours prior to infection with a single subcutaneous injection of clindamycin at 10 or 30 mg/kg. On the day of infection, animals are inoculated by oral gavage with 10^6 *C. difficile* (ATCC 43255) vegetative cells per hamster. The *C. difficile* inoculum is prepared by growing the bacteria in Difco reinforced clostridial medium with 1% Oxyrase for 24 hours under anaerobic conditions. The optical density at 600 nm is adjusted to 1.5 and then diluted 1:10. The hamsters are given 0.75 ml of this suspension orally via gavage. An aliquot of the inoculum is then serially diluted, plated on brucella agar supplemented with hemin and vitamin K_1 (Remel, Lenexa, KS), and incubated anaerobically for 48 hrs in an airtight container (Pack-Anaero MGC) to determine the infection titer. Animals are observed twice daily during the first 24 hrs post-infection and then every 2 hours for the following 24 hours during the acute phase of the disease, followed by twice daily for the remainder of the study. Signs of CDI include signs of mortality and morbidity, presence of diarrhea as indicated by a wet tail, and overall appearance including activity, general response to handling, touch, or ruffled fur. Body weights are monitored every 2 to 3 days.

[0158] To evaluate the prophylactic potential of the S. *boulardii* transformants expressing the *C. difficile* anti-Toxin A and/or anti-Toxin B VHHs, the yeast are administered, via oral gavage, at doses ranging from 100 to 500 mg, approximately 2 x 10^8 to 10^{10} cfu/animal daily beginning at the time of clindamycin administration, 5 or 1 day prior to *C. difficile* infection, and continued for the duration of the studies, up to 28 days. As yeast are not

sensitive to clindamycin, the yeast will remain viable even in the presence of antibiotics. The efficacy of the VHH-expressing yeast are compared to control animals that receive no treatment, animals that receive the standard of care, vancomycin (20 mg/kg orally daily beginning 24 hrs after infection and continued for 5 days), animals that receive both vancomycin and the yeast, and/or animals that receive wild type yeast that do not express the *C. difficile* anti-Toxin A and/or anti-Toxin B VHHs. Efficacy evaluations include mortality and evaluation of *C. difficile* bacteria titers and/or *C. difficile* toxins A and B in cecal contents, at the time of death or at the end of the study following euthanasia. Additional evaluations may include cytokine levels and histopathology. *See* Table 6 for the experimental design.

[0159] To evaluate the therapeutic potential of the S. *boulardii* transformants expressing the *C. difficile* anti-Toxin A or anti-Toxin B VHHs, the yeast are administered, via oral gavage, at doses ranging from 100 to 500 mg, approximately 2 x 10^8 to 10^{10} cfu/animal daily beginning when disease symptoms first appear or 24 hrs after infection, as the acute stage of the disease occurs within 48 hrs of infection. Yeast administration is continued daily for the duration of the study, up to 28 days. The efficacy of the VHH-expressing yeast are compared to control animals that receive no treatment, animals that receive the wildtype, non-transformed yeast, animals that receive the standard of care, vancomycin (20 mg/kg orally daily beginning 24 hrs after infection and continued for 5 days), or animals that receive both vancomycin and the yeast, as yeast are not killed by antibiotics. Efficacy evaluations include mortality and evaluation of *C. difficile* bacteria titers and/or *C. difficile* toxins A and B in cecal contents, at the time of death or at the end of the study following euthanasia. Additional evaluations may include cytokine levels and histopathology. *See* Table 6 for an experimental design.

[0160] Similar studies are performed to assess the prophylactic and therapeutic potential of *Pichia pastoris* transformants expressing the *C. difficile* anti-Toxin A or anti-Toxin B VHHs using the rodent models as described herein.

[0161] Table 6. *C. difficile* efficacy hamster study treatment groups (for prophylactic and therapeutic efficacy studies)

Cohort (n=8-10)	Treatment
1	Oral Saline
2	Vancomycin (standard of care)
3	Wild-type S. *boulardii*
4	Anti-Toxin A S. *boulardii*
5	Anti-toxin B S. *boulardii*
6	Combination of Anti-toxin A and Anti-toxin B S. *boulardii*
7	Combination of Anti-toxin A and Anti-toxin B S. *boulardii* plus vancomycin

Example 6: *In Vivo* Analysis of Yeast Expressed VHHs in Porcine Models of *C. difficile* Disease

[0162] The S. *boulardii* transformants expressing the *C. difficile* anti-Toxin A and/or anti-Toxin B VHHs are evaluated in two pig models of *C. difficile* disease (CDI), the gnotobiotic piglet model and the humanized piglet model. The gnotobiotic piglet model, when exposed to *C. difficile,* is a model for acute or chronic CDI including pseudomembranous colitis (Steele *et al.*, 2010; J. Infect. Dis 201:428). The humanized pig model is a model of the human gastrointestinal tract where the gnotobiotic pigs are reconstituted with human fecal homogenates (Zhang *et al.*, Gut Microbes 4:193). The humanized pigs are treated with antibiotics (*e.g.* clindamycin or ceftriaxone) to disrupt their intestinal microbiome and then exposed to *C. difficile* after which they develop CDI including *C. difficile* associated diarrhea (CDAD). Either model can be used to evaluate the *C. difficile* anti-Toxin secreting S. *boulardii* as prophylactic and/or therapeutic treatments for CDI.

[0163] For the gnotobiotic piglet studies, to test the prophylactic potential of the *C. difficile* anti-Toxin secreting S. *boulardii,* the yeast are delivered orally, at a dose range of 250 mg to 3000 mg/animal, approximately 5 x 10^9 to 6 x 10^{10} cfu/animal , once daily beginning 3-7 days prior to *C. difficile* exposure. Animals are then exposed to *C. difficile*, vegetative cells or spores, at doses ranging from 10^6 to 10^8, and monitored for CDI symptoms including CDAD. Animals exposed to *C. difficile* are expected to develop disease symptoms within 48 hrs of bacterial inoculation (Steele *et al.*, 2010; J. Infect. Dis 201:428).

[0164] To test the therapeutic potential of the *C. difficile* anti-Toxin secreting S. *boulardii,* the animals are inoculated with *C. difficile* vegetative cells or spores and allowed to develop symptoms of CDI, 24-48 hours after inoculation, after which they are treated orally with the transformed S. *boulardii* daily at a dose range of 250 mg to 3000 mg/animals, approximately 5 x 10^9 to 6 x 10^{10} cfu/animal. Animals are monitored for disease symptoms. As the yeast are not killed by antibiotics, the yeast can be tested in the presence of antibiotics used in the standard of care for CDI, including vancomycin and metronidazole (Cohen *et al.*, 2010. Infect. Control Hosp. Epidemiol. 31:431). Importantly, vancomycin and metronidazole are effective in inhibiting the growth of the *C. difficile* pathogen during therapy, however, they often fail to prevent recurrence of disease after treatment is completed. The use of the *C. difficile* anti-Toxin secreting S. *boulardii* combined with the standard of care antibiotic therapy may show efficacy in preventing reoccurrence of CDI. Therefore cohorts of animals treated with vancomycin and the yeast are added to the study. These gnotobiotic pig studies include 7 cohorts (n=2) as described in Table 7.

[0165] For the piglet studies, the *C. difficile* strain UK6, a type 027/BI21/NAP1, which produced *C. difficile* Toxin A, Toxin B, and binary toxin is used as described (Steele *et al.*, 2010; J. Infect. Dis 201:428). Briefly, vegetative cells for the inocula are grown anaerobically overnight in pre-reduced brain heart infusion (BHI) broth at 37°C. The concentration is adjusted to contain 10^8 CFU per 2 ml per piglet. Spores are grown on pre-reduced BHI agar plates anaerobically at 37°C for 48 hrs. Colonies, scraped off the plates, were suspended in BHI broth, left in flasks for 7-10 days in an anaerobic chamber at 37°C to induce sporulation. The suspension is centrifuged, supernatant discarded, and washed with sterile PBS twice. The suspension is then heated at 70°C for 20

minutes to kill vegetative cells. The spore suspension is stored at 4°C and spore concentration is determined by serial dilution before use.

Table 7. Gnotobiotic pig study treatment groups (for prophylactic and therapeutic efficacy studies)

Cohort (n=2)	Treatment
1	Oral Saline
2	Standard of care (*e.g.*, vancomycin)
3	Wild-type S. *boulardii*
4	Anti-Toxin A S. *boulardii*
5	Anti-toxin B S. *boulardii*
6	Combination of Anti-toxin A and Anti-toxin B S. *boulardii*
7	Combination of Anti-toxin A and Anti-toxin B S. *boulardii* plus Standard of care

[0166] The humanized pig model of CDI is also used to evaluate the *C. difficile* anti-Toxin secreting S. *boulardii* as a therapeutic agent. To test the prophylactic potential of the *C. difficile* anti-Toxin secreting S. *boulardii,* the yeast are delivered orally, at a dose range of 250 mg to 3000 mg/animal, approximately 5 x 10^9 to 6 x 10^{10} cfu/animal, once daily beginning 3-7 days prior to antibiotic treatment to gnotobiotic pigs that have been reconstituted with a human fecal microbiome (Zhang *et al.*, Gut Microbes 4:193). Yeast treatment is continued for the duration of the study. Antibiotic (clindamycin, ceftriaxone, or another antibiotic) is delivered 1 to 5 days prior to *C. difficile* inoculation, to disrupt the intestinal microbiome, and then animals are exposed to *C. difficile* vegetative cells or spores as described. The animals are monitored for symptoms of CDI including CDAD.

[0167] To test the therapeutic potential of the *C. difficile* anti-Toxin secreting S. *boulardii* in the humanized pig model of CDI, CDI is established in the humanized pigs by treatment with antibiotics (*e.g.* clindamycin, ceftriaxone, or another antibiotic) 1-5 days prior to exposure to *C. difficile* vegetative cells or spores. The animals are monitored for symptoms of CDI, including CDAD. Once disease symptoms are present, anticipated to be 24 to 48 hours after *C. difficile* inoculation, animals are treated with the *C. difficile* anti-Toxin secreting S. *boulardii,* delivered orally, at a dose range of 250 mg to 3000 mg/animal, approximately 5 x 10^9 to 6 x 10^{10} cfu/animal, once daily for the duration of the study. In all studies using the humanized pigs, the *C. difficile* anti-Toxin secreting S. *boulardii* can be used in combination with the standard of care, including vancomycin and metronidazole (Cohen *et al.*, 2010. Infect. Control Hosp. Epidemiol. 31:431), as the yeast are not killed by antibiotics. Importantly, vancomycin and metronidazole are effective in inhibiting the growth of the *C. difficile* pathogen during therapy, however, they often fail to prevent recurrence of disease after treatment is completed. The use of the *C. difficile* anti-Toxin secreting S. *boulardii* combined with the standard of care antibiotic therapy may show efficacy in preventing reoccurrence of CDI. These humanized pig studies include 8 cohorts (n=2) as described in Table 8.

[0168] Similar studies are performed to evaluate the prophylactic and therapeutic potential of *Pichia pastoris* transformants expressing the *C. difficile* anti-Toxin A or anti-Toxin B VHHs using the pig models as described herein.

[0169]

Table 8. Humanized pig study treatment groups

Cohort (n=2)	Antibiotic Treatment (IV or oral)	C. diff innoculation	S. *boulardii* treatment	Standard of care treatment
1	None	None	None	None
2	clindamycin	+	None	None
3	clindamycin	+	Wild-type	None
4	clindamycin	+	Anti-Toxin A	None
5	clindamycin	+	Anti-Toxin B	None
6	clindamycin	+	Combination, Anti-Toxin A + Anti-Toxin B	None
7	clindamycin	+	None	vancomycin
8	clindamycin	+	Combination, Anti-Toxin A + Anti-Toxin B	vancomycin

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EQUIVALENTS

[0170] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

[0171] Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

INCORPORATION BY REFERENCE

[0172] All patents and publications referenced herein are hereby incorporated by reference in their entireties.

[0173] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[0174] As used herein, all headings are simply for organization and are not intended to limit the disclosure in any manner. The content of any individual section may be equally applicable to all sections.

CLAIMS

What is claimed is:

1. A recombinant yeast cell capable of producing at least one binding protein for neutralizing a *C. difficile* toxin,

wherein the binding protein includes two or more binding regions, and

wherein the binding regions are not identical and each binding region binds to a non-overlapping portion of the *C. difficile* toxin.

2. The recombinant yeast cell of claim 1, wherein the yeast cell is selected from *Saccharomyces* sp., *Hansenula* sp., *Kluyveromyces* sp. *Schizzosaccharomyces* sp. *Zygosaccharoinyces* sp., *Pichia* sp., *Monascus* sp., *Geotrichum* sp and *Yarrowia* sp.

3. The recombinant yeast cell of claim 2, wherein the *Saccharomyces* sp. is *Saccharomyces cerevisiae*.

4. The recombinant yeast cell of claim 3, wherein the recombinant yeast cell is *Saccharomyces cerevisiae* subspecies *Saccharomyces boulardii*.

5. The recombinant yeast cell of claim 2, wherein the recombinant yeast cell is *Pichia pastoris*.

6. The recombinant yeast cell any one of the above claims, wherein the binding protein neutralizes toxin A.

7. The recombinant yeast cell any one of the above claims, wherein the binding protein neutralizes toxin B.

8. The recombinant yeast cell of any one of the above claims, wherein the binding protein neutralizes both toxin A and toxin B.

9. The recombinant yeast cell of any one of the above claims, wherein the binding protein comprises a recombinant camelid heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', or a $F(ab')_2$.

10. The recombinant yeast cell of claim 9, wherein the binding protein comprises a recombinant camelid heavy-chain-only antibody (VHH).

11. The recombinant yeast cell of any of the above claims, wherein the binding region binds to a portion of *C. difficile* toxin A and comprises an amino acid sequence that is at least 60% identical to SEQ ID NOs: 1-8.

12. The recombinant yeast cell of any of the above claims, wherein the binding region binds to a portion of *C. difficile* toxin B and comprises an amino acid sequence that is at least 60% identical to SEQ ID NOs: 9-20.

13. The recombinant yeast cell of claims 11 and 12, wherein the binding region comprises an amino acid sequence having at least one amino acid alteration with respect to SEQ ID NOs: 1-20.

14. The recombinant yeast cell of claim 13, wherein the amino acid alteration is an amino acid substitution, deletion, or insertion.

15. The recombinant yeast of cell of claim 13, wherein the amino acid alternation is an amino acid substitution.

16. The recombinant yeast cell of any one of claims 13-15, wherein the amino acid alteration is in a joining region.

17. The recombinant yeast cell of any one of the above claims, wherein the binding regions are separated by a linker.

18. The recombinant yeast cell of claim 17, wherein the linker is a peptide or a protein.

19. The recombinant yeast cell of claim 18, wherein the linker is a peptide.

20. The recombinant yeast cell of claim 19, where the peptide comprises an amino acid sequence that is protease-resistant.

21. The recombinant yeast cell of claim 20, wherein the peptide comprises the sequence GGGGSGGGGSGGGGS (SEQ ID NO: 22) or a portion thereof.

22. The recombinant yeast cell of any of the above claims, wherein the neutralizing binding protein further includes at least one tag that is an epitope recognized by an antibody.

23. A replicable yeast expression vector comprising a nucleic acid encoding the binding protein of claim 1.

24. A pharmaceutical composition comprising the recombinant yeast cell of any one of the above claims and a pharmaceutically acceptable carrier and/or excipient.

25. The pharmaceutical composition of claim 24, wherein the pharmaceutical composition is formulated for intestinal delivery.

26. The pharmaceutical composition of claim 25, wherein the recombinant yeast cell is released in the small intestine.

27. The pharmaceutical composition of claim 25, wherein the recombinant yeast cell is released in the large intestine.

28. The pharmaceutical composition of any one of claims 24-27, wherein the composition is formulated for oral administration.

29. The pharmaceutical composition of any one of claims 24-28, wherein the composition is formulated as a capsule or a tablet.

30. The pharmaceutical composition of any one of claims 24-29, further comprising an additional therapeutic agent.

31. The pharmaceutical composition of claim 30, wherein the additional therapeutic agent is a beta-lactamase.

32. A method of treating or preventing *C. difficile* infection (CDI) and/or a *C. difficile*-associated disease, comprising administering an effective amount of a pharmaceutical composition of any one of the above claims to a subject.

33. The method of claim 32, wherein the *C. difficile* infection or *C. difficile*-associated disease is one or more of: *C. difficile* diarrhea (CDD), *C. difficile* intestinal inflammatory disease, colitis, pseudomembranous colitis, and/or peritonitis.

34. The method of claim 32 or 33, wherein the *C. difficile* infection and/or *C. difficile* associated disease is treated in the context of initial onset or relapse.

35. The method of any one of claims 32-34, wherein the subject is undergoing treatment or has recently undergone treatment with one or more primary antibiotic.

36. The method of any one of claims 32-35, wherein an initial and/or adjunctive therapy is administered to a subject.

37. The method of claim 36, wherein the initial and/or adjunctive therapy is one or more of metronidazole, vancomycin, fidaxomicin, rifaximin, fecal bacteriotherapy, probiotic therapy, and antibody therapy.

FIGURE 1

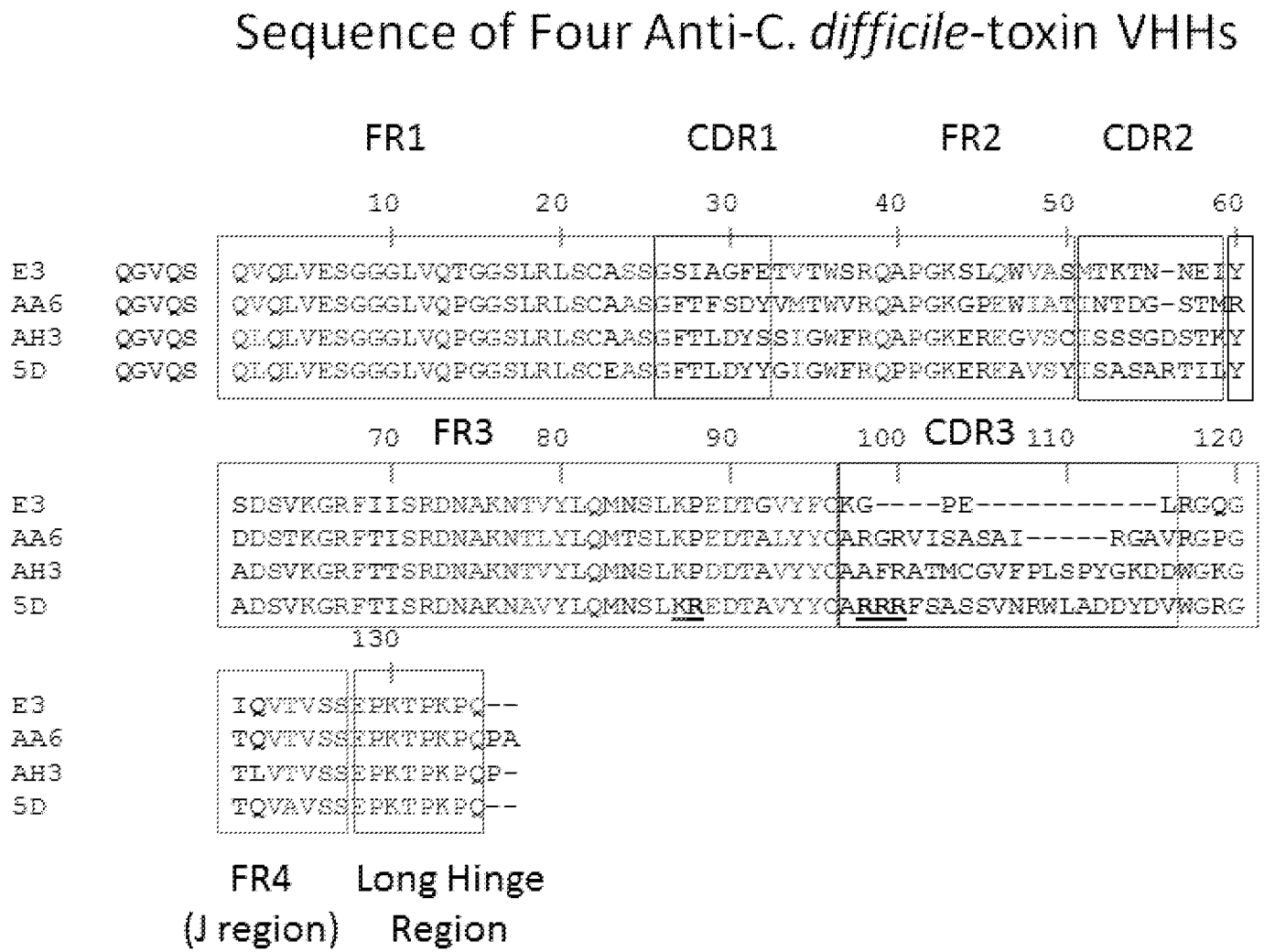

Sequence of Four Anti-C. difficile-toxin VHHs
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
(J region)
Long Hinge
Region
E3
AA6
AH3
5D

FIGURE 2

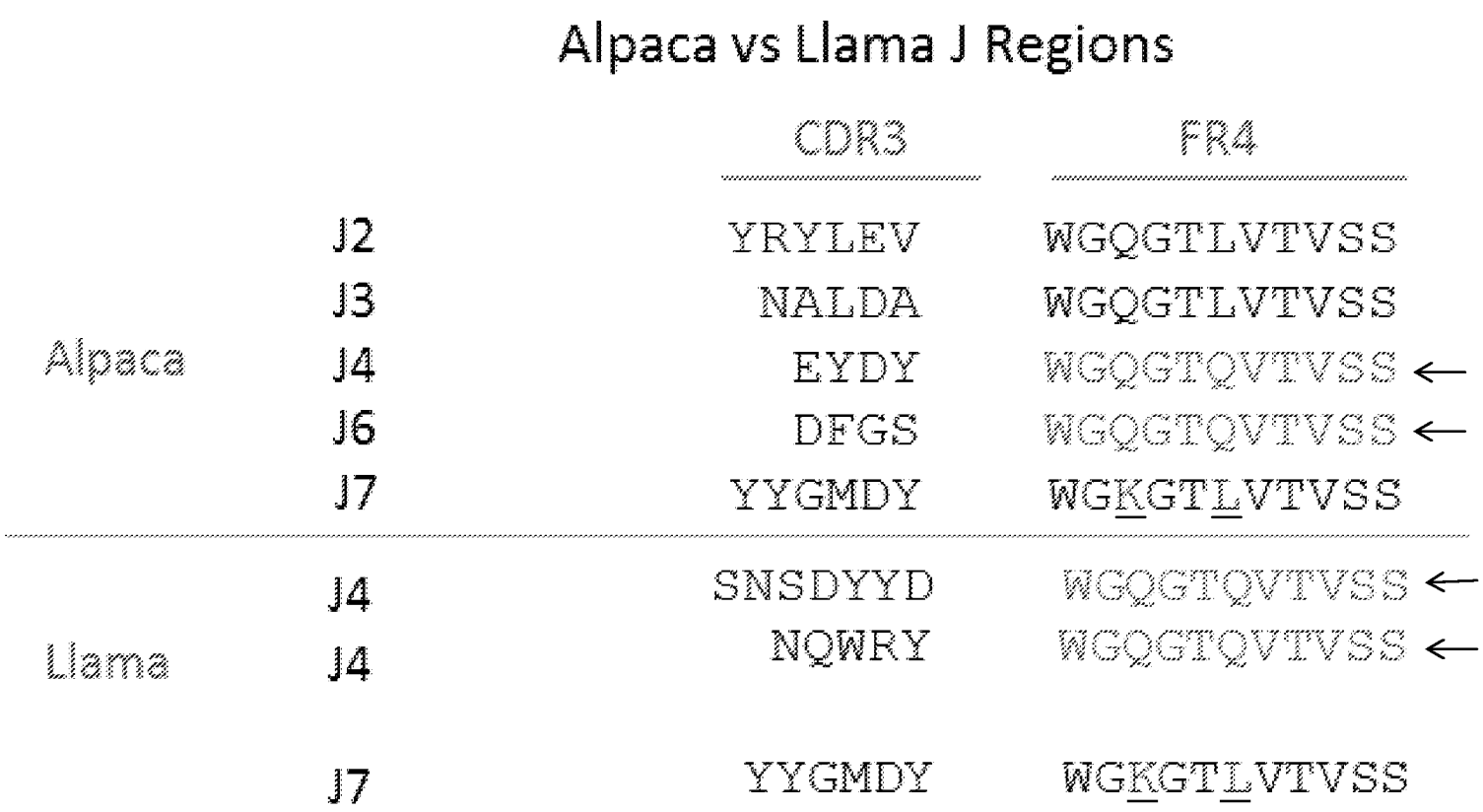

Alpaca vs Llama J Regions
CDR3
FR4
Alpaca
J2
YRYLEV
WGQGTLVTVSS
J3
NALDA
WGQGTLVTVSS
J4
EYDY
WGQGTQVTVSS
J6
DFGS
WGQGTQVTVSS
J7
YYGMDY
WGKGTLVTVSS
Llama
J4
SNSDYYD
WGQGTQVTVSS
J4
NQWRY
WGQGTQVTVSS
J7
YYGMDY
WGKGTLVTVSS

FIGURE 3

Comparison of C. diff anti-Toxin VHH J regions to J4 region

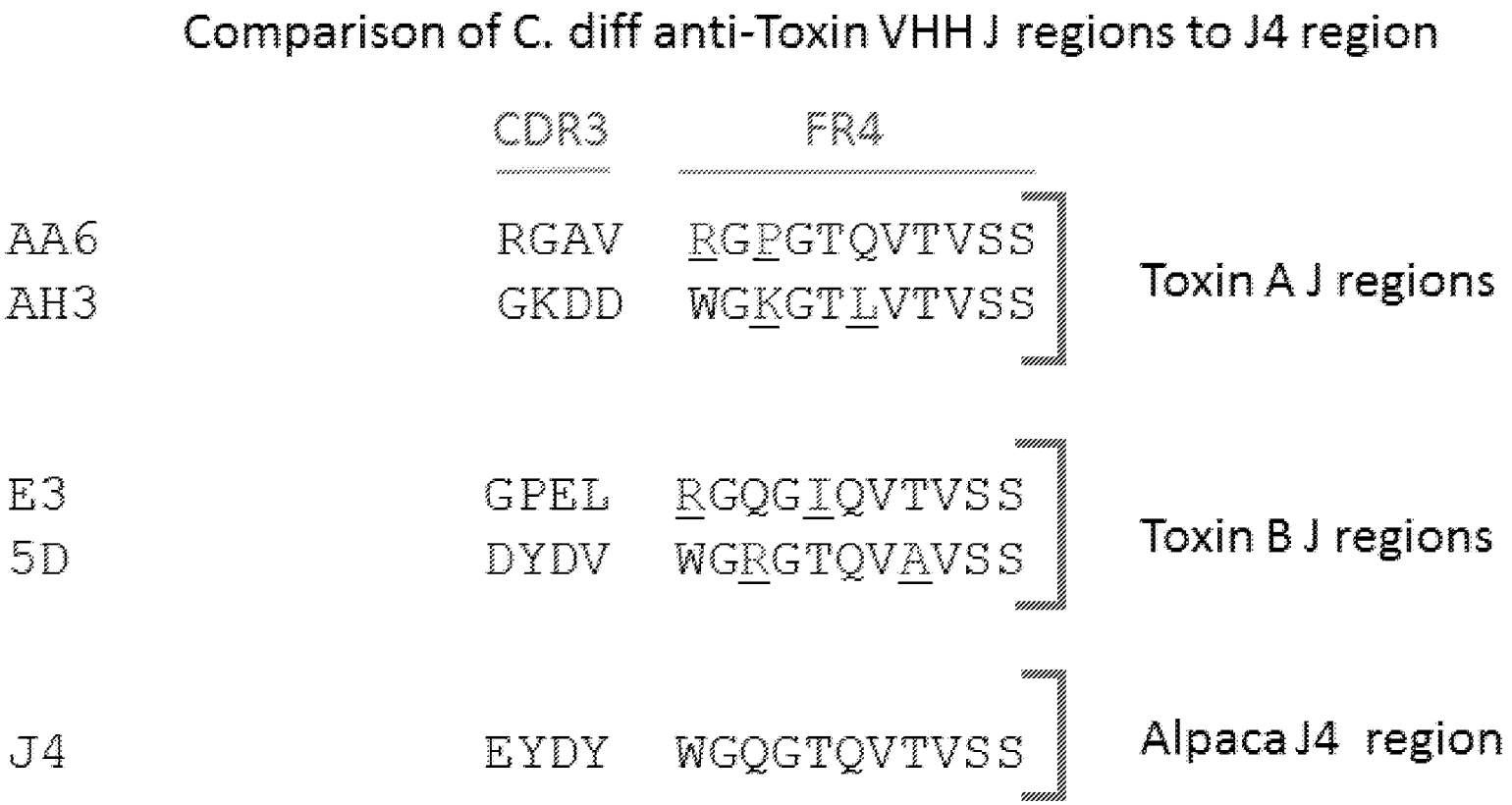


FIGURE 4

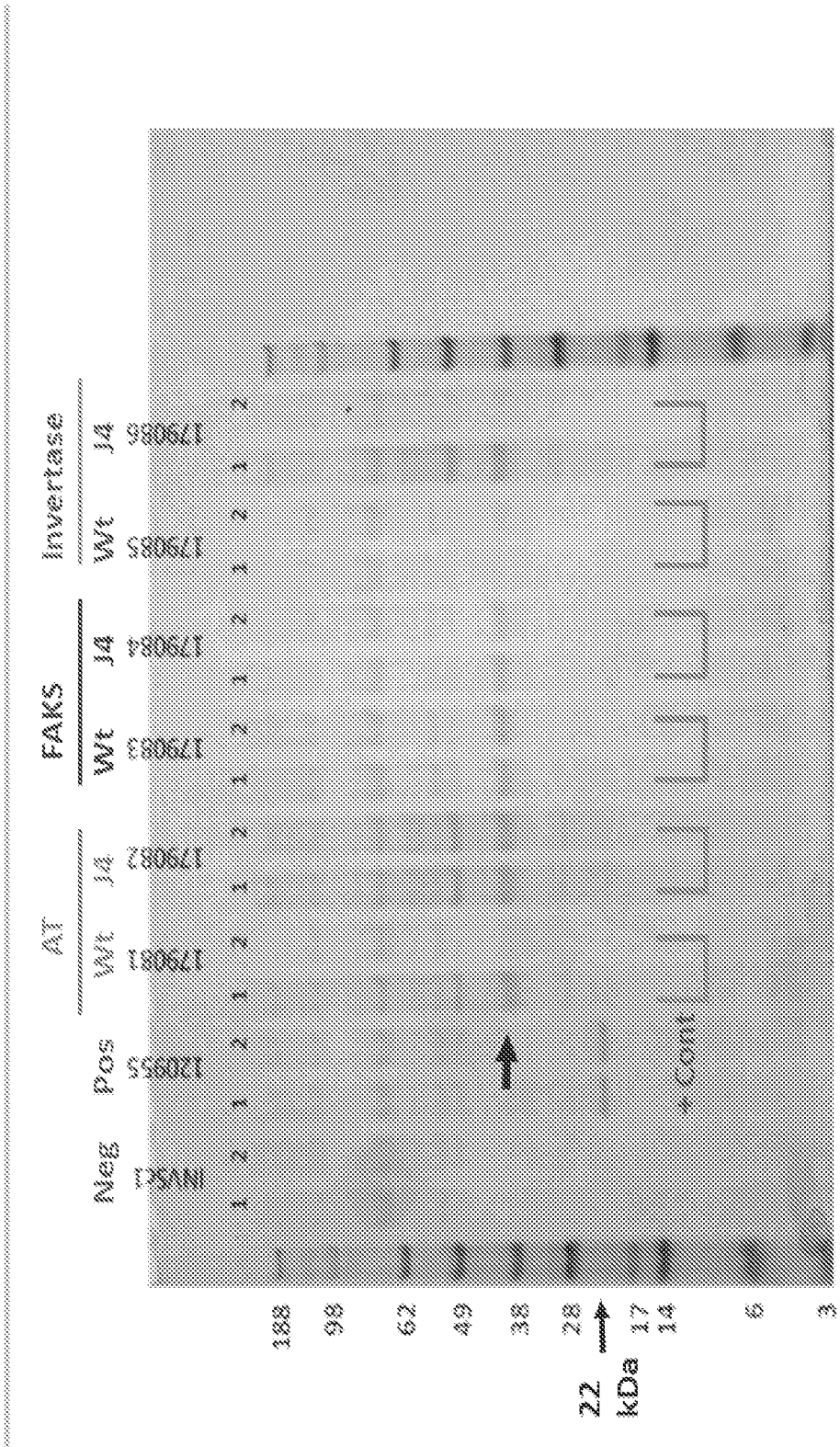

Yeast Expression: AA SDS PAGE
AT
FAKS
Invertase
Neg
Pos
Wt
J4
Wt
J4
Wt
J4
1
2
188
98
62
49
38
28
17
14
6
3
22 kDa
+ Con

FIGURE 5

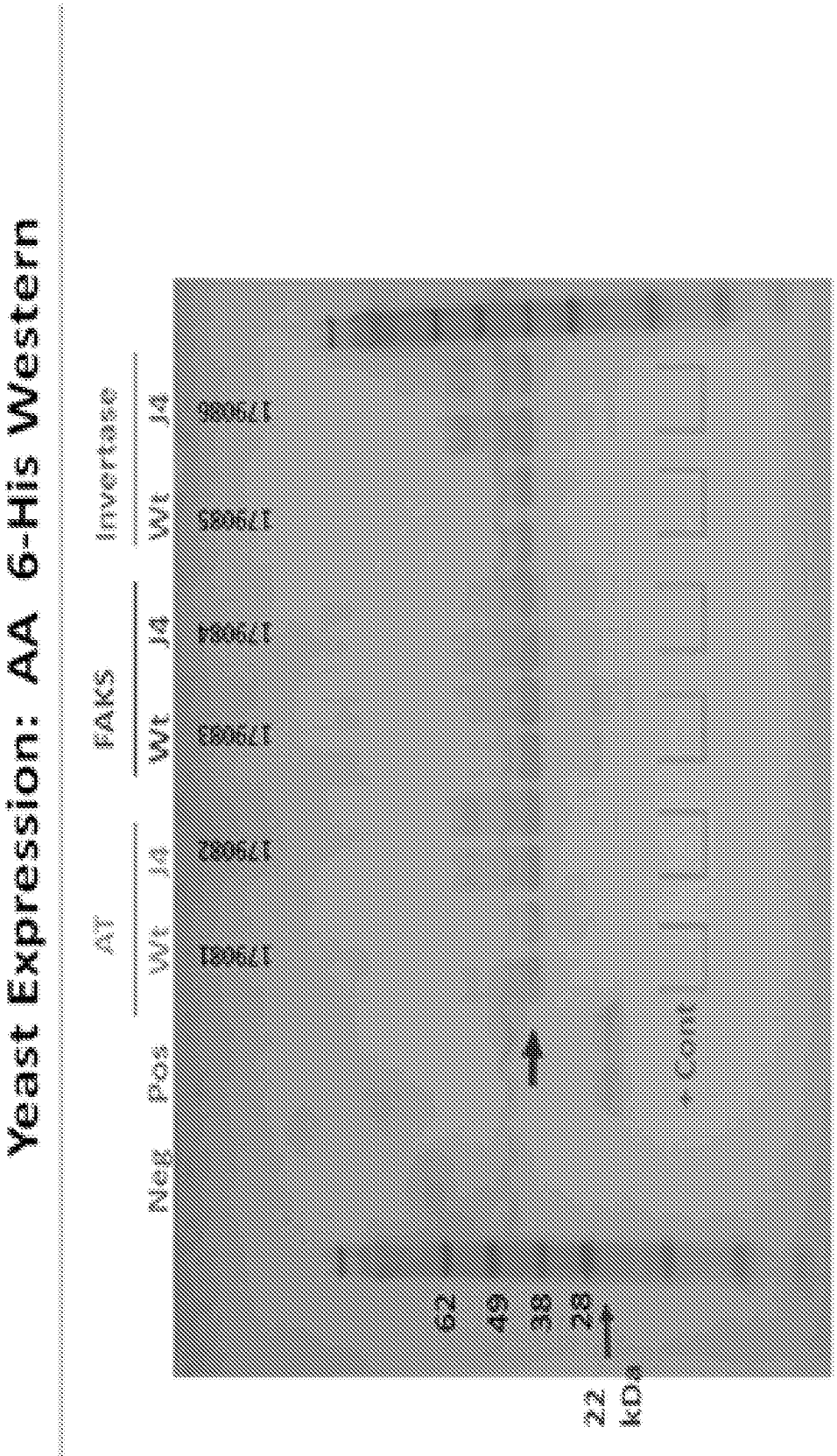

Yeast Expression: AA 6-His Western
AT
FAKS
Invertase
Neg
Pos
Wt
J4
Wt
J4
Wt
J4
179081
179082
179083
179084
179085
179086
62
49
38
28
22
kDa
FAKS

FIGURE 6

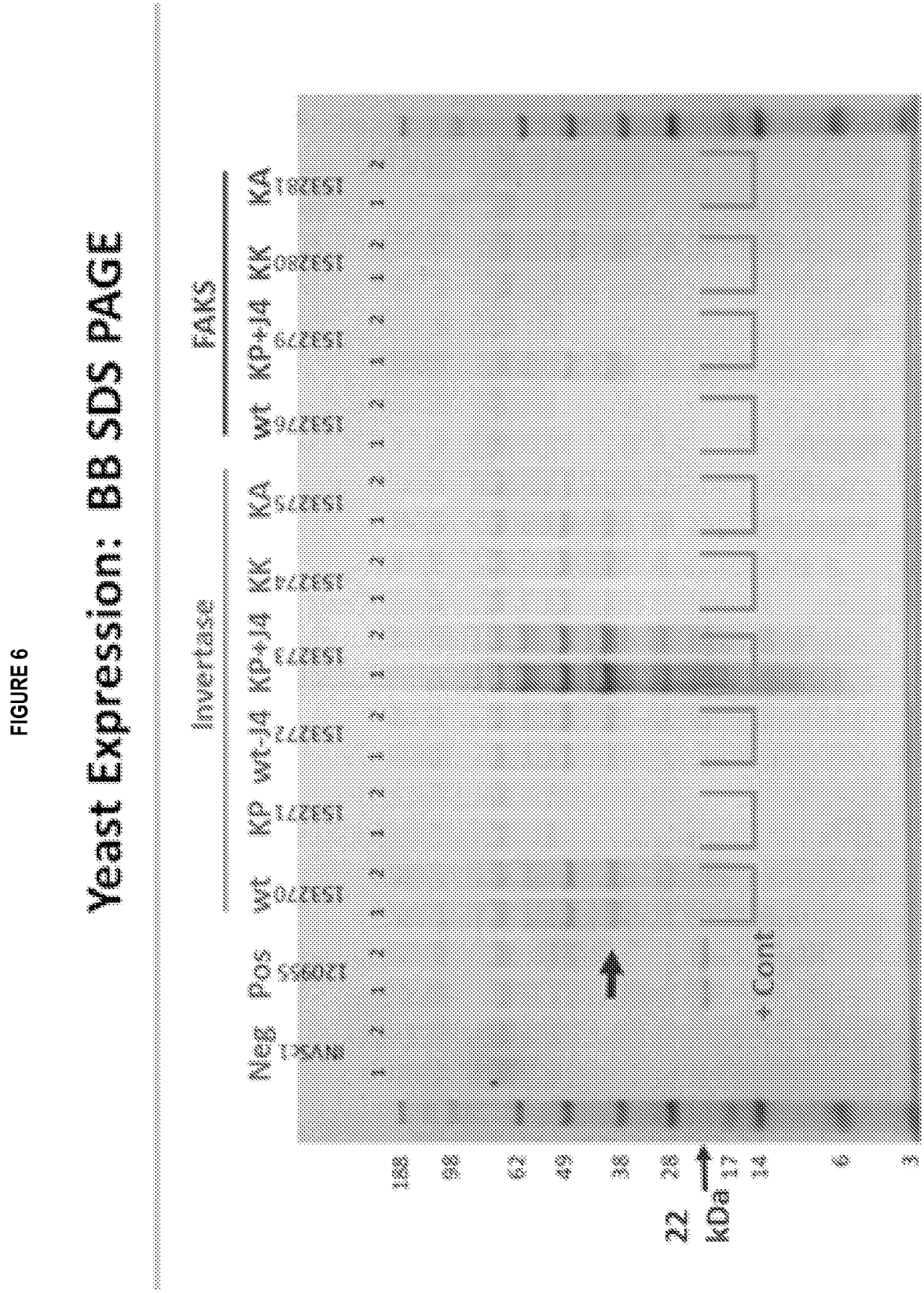

Yeast Expression: BB SDS PAGE
Invertase
FAKS
Neg
Pos
wt
KP
wt-J4
KP+J4
KK
KA
1%SAN
SSBOCL
1S1270
1S1271
1S1272
1S1273
1S1274
1S1275
1S1276
1S1277
1S1278
1S1279
1S1280
1S1281
+ Cont
188
98
62
49
38
28
17
14
6
3
22
kDa

FIGURE 7

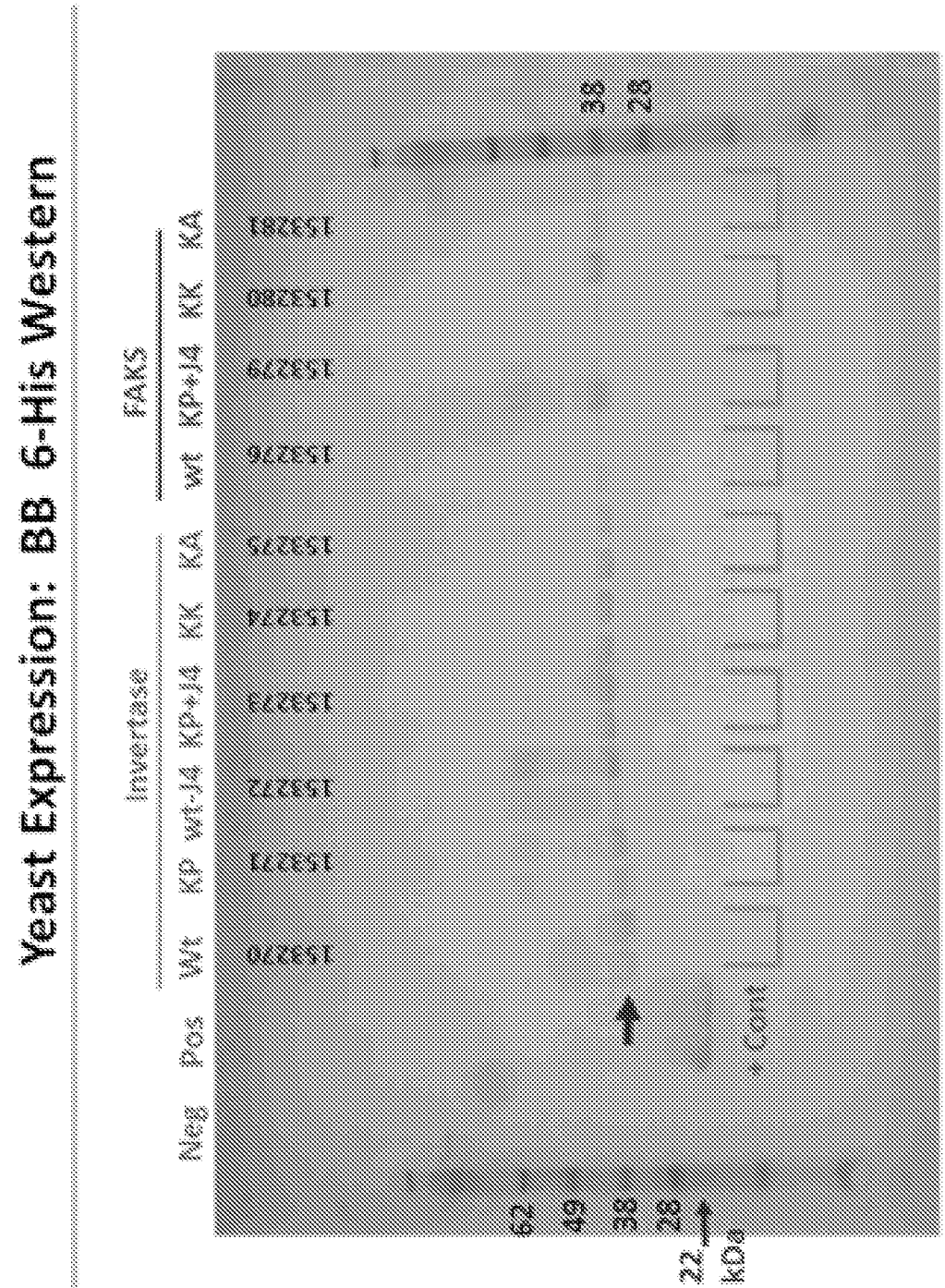

Yeast Expression: BB 6-His Western
Invertase
FAKS
Neg
Pos
Wt
KP
wt-J4
KP+J4
KK
KA
wt
KP+J4
KK
KA
62
49
38
28
22
kDa
38
28
+ Con

FIGURE 8

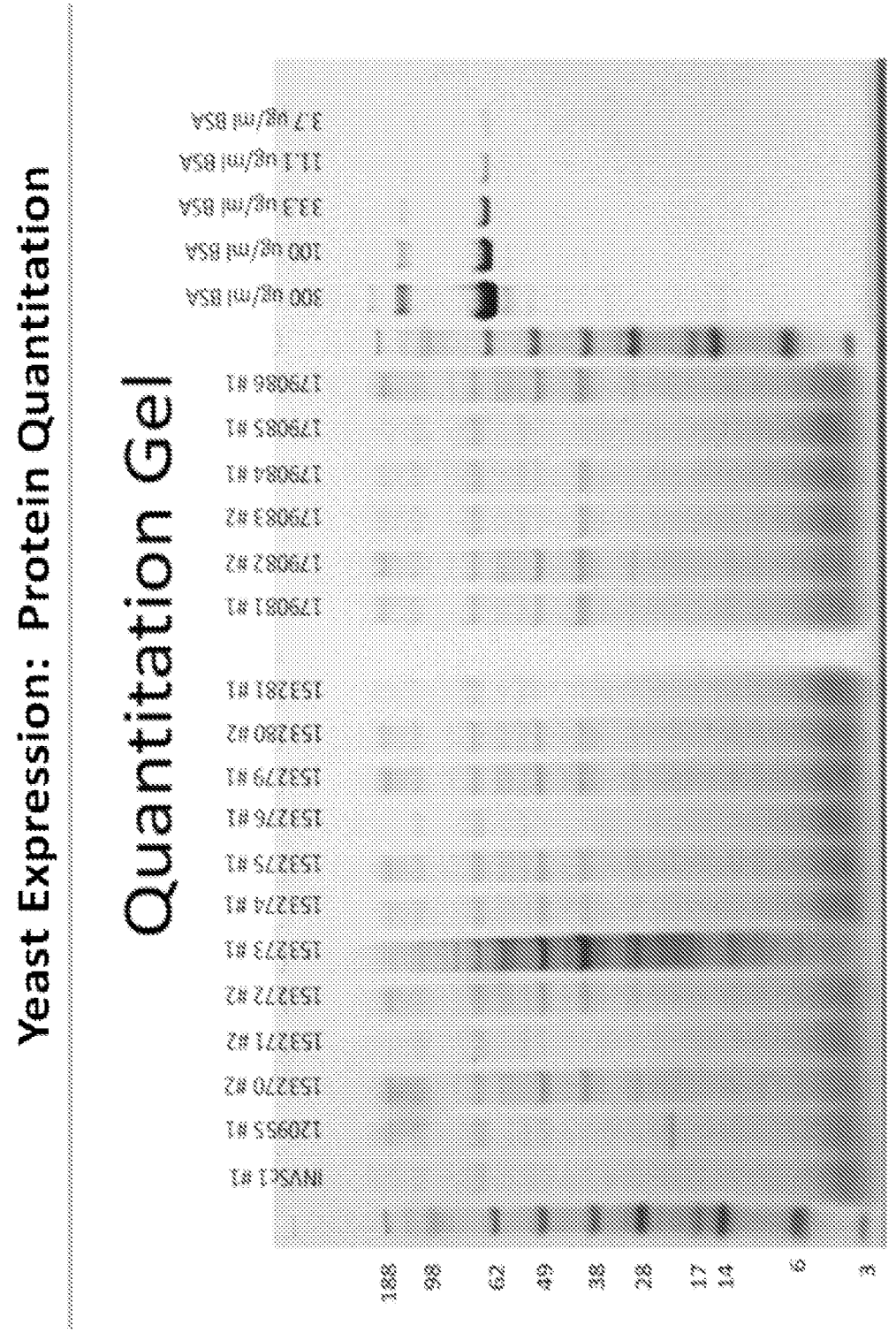

Yeast Expression: Protein Quantitation
Quantitation Gel
188
98
62
49
38
28
17
14
6
3
NVSc1 #1
120855 #1
153270 #2
153271 #2
153272 #2
153273 #1
153274 #1
153275 #1
153276 #1
153279 #1
153280 #2
153281 #1
125081 #1
125082 #2
125083 #2
125084 #1
125085 #1
125086 #1
300 ug/ml BSA
100 ug/ml BSA
33.3 ug/ml BSA
11.1 ug/ml BSA
3.7 ug/ml BSA

FIGURE 9

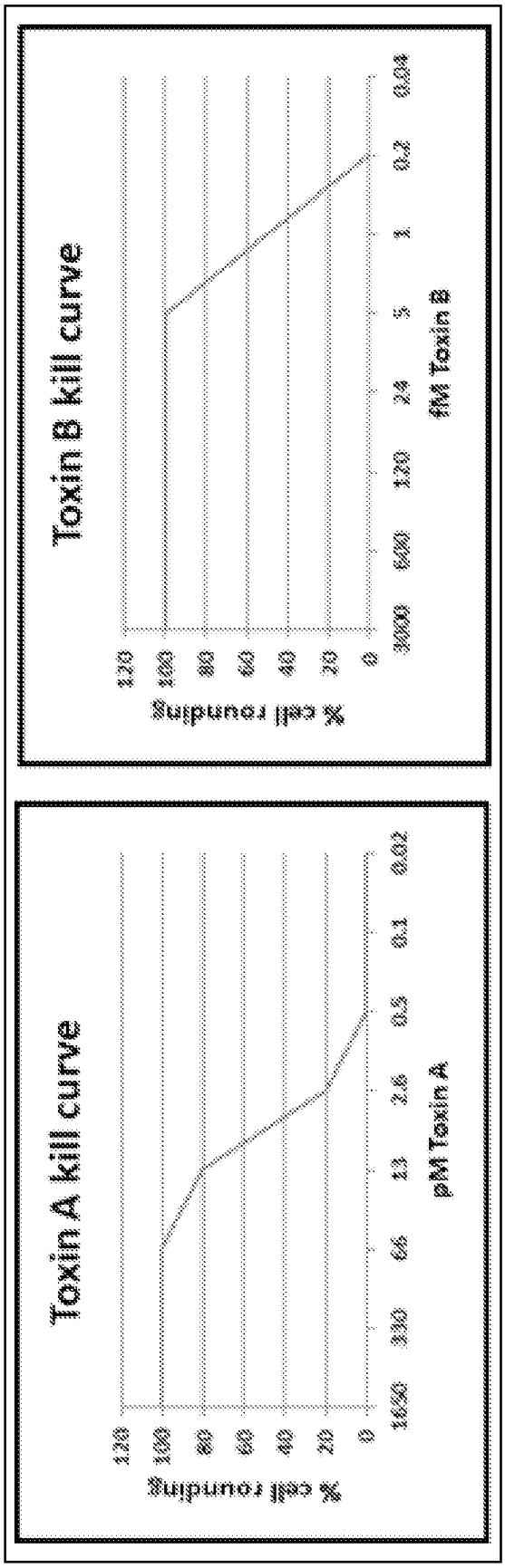

Toxin A kill curve
% cell rounding
120
100
80
60
40
20
0
1650
330
66
13
2.6
0.5
0.1
0.02
pM Toxin A
Toxin B kill curve
% cell rounding
120
100
80
60
40
20
0
3000
600
120
24
5
1
0.2
0.04
fM Toxin B

FIGURE 10

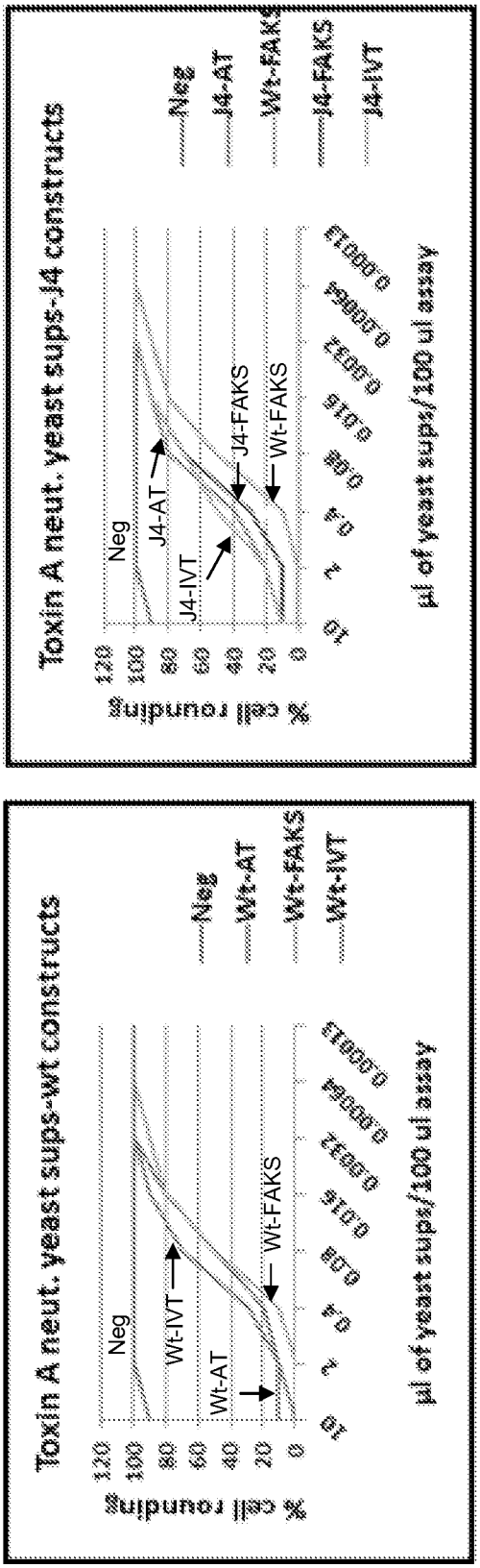
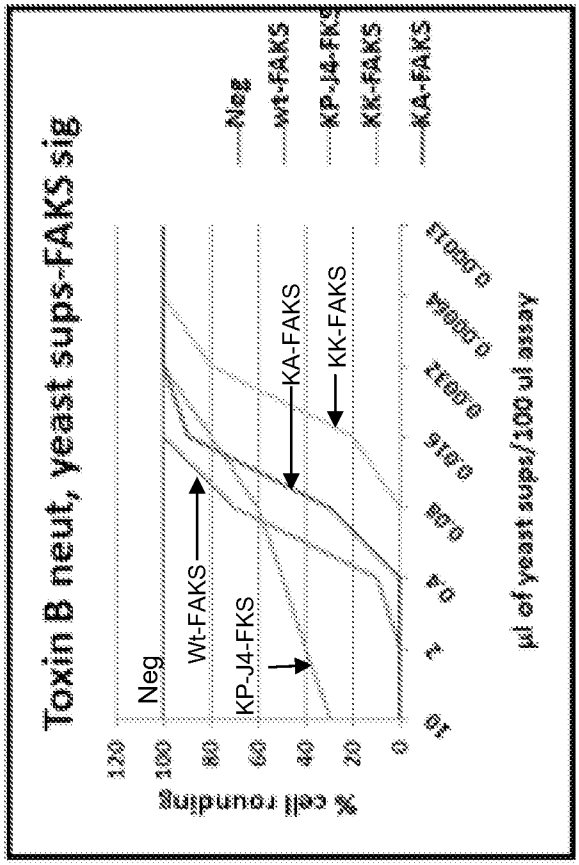
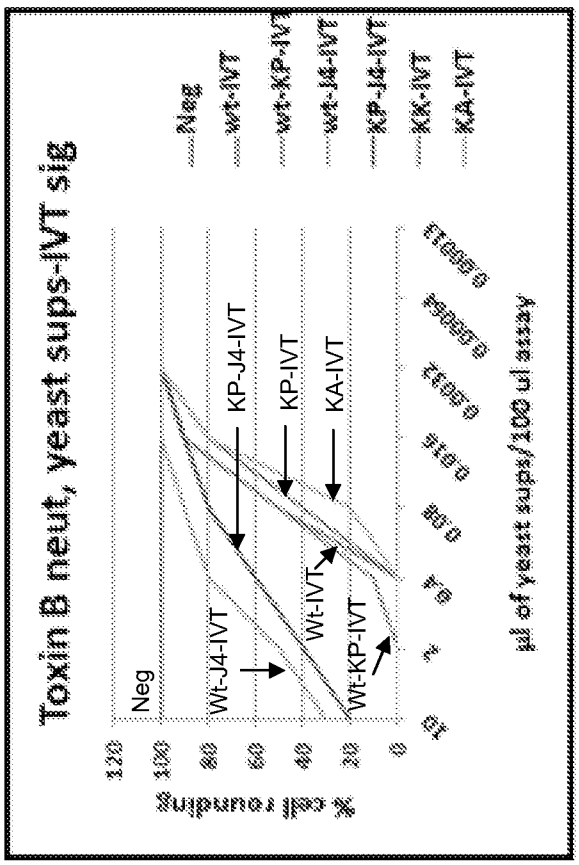

Toxin A neut. yeast sups-wt constructs
% cell roundins
120
100
80
60
40
20
0
Neg
Wt-IVT
Wt-AT
Wt-FAKS
1
2
4
8
16
32
64
128
256
512
µl of yeast sups/100 ul assay
Neg
Wt-AT
Wt-FAKS
Wt-IVT
Toxin A neut. yeast sups-J4 constructs
% cell roundins
120
100
80
60
40
20
0
Neg
J4-AT
J4-IVT
J4-FAKS
Wt-FAKS
1
2
4
8
16
32
64
128
256
512
µl of yeast sups/100 ul assay
Neg
J4-AT
J4-FAKS
J4-IVT

FIGURE 11


Toxin B neut, yeast sups-FAKS sig
% cell rounding
120
100
80
60
40
20
0
Neg
Wt-FAKS
KP-J4-FKS
KA-FAKS
KK-FAKS
Neg
wt-FAKS
KP-J4-FKS
KK-FAKS
KA-FAKS
µl of yeast sups/100 ul assay


Toxin B neut, yeast sups-IVT sig
% cell rounding
120
100
80
60
40
20
0
Neg
Wt-J4-IVT
KP-J4-IVT
KP-IVT
Wt-IVT
KA-IVT
Wt-KP-IVT
Neg
wt-IVT
wt-KP-IVT
wt-J4-IVT
KP-J4-IVT
KK-IVT
KA-IVT
µl of yeast sups/100 ul assay

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/58967

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53, C12P 1/04, C12N 15/00 (2015.01)
CPC - A61K 38/00, A61K 35/74, C12N 15/86
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)
IPC(8): G01N 33/53, C12P 1/04, C12N 15/00 (2015.01)
CPC: A61K 38/00, A61K 35/74, C12N 15/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/7.1, 435/170, 435/320.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Yeast, recombinant*, antibod*, difficile, toxin, saccharomyces, cerevisiae, pastoris, boulardii, recombinant yeast, neutralizing, binding, producingPubWest, PatBase, Google Scholar, Google Patents:

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0087478 A1 (Hansen et al.) 02 April 2009 (02.04.2009) para [0076], [0263], [0264]	1-6, 23
Y	DEMAREST et al., Neutralization of Clostridium difficile toxin A using antibody combinations, Landes Bioscience, April 2010, Vol. 2, No. 2; pg 190-198; pg 192, col 1, para 2, Fig 3	1-6, 23
Y	US 2011/0183348 A1 (Hyde et al.) 28 July 2011 (28.07.2011) abstract, [0219], [0380]	4, 6/4, 23

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
29 December 2015 (29.12.2015)	27 JAN 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/58967

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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.: 7-22, 24-37
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 additional fees, this Authority did not invite payment of 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.