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(54) **Title**: ANTIBODIES TO CLOSTRIDIUM DIFFICILE SPORES AND USES THEREOF

(57) **Abstract**: The present invention provides antibodies that bind to the endospore of the bacterium Clostridium difficile, methods of making such antibodies, and methods of using such antibodies, including methods of detecting C. difficile endospores.

ANTIBODIES TO CLOSTRIDIUM DIFFICILE SPORES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/032,270, filed February 28, 2008, which is incorporated herein by reference.

BACKGROUND

Clostridium difficile, an anaerobic spore forming Gram-positive bacteria, is the major cause of pseudomembraneous colitis and antibiotic associated diarrhea in humans and is one of the most widespread bacterium implicated in hospital acquired, nosocomial infections (see, for example, Wren, 2006, *Future Microbiol*; 1(3):243-245). According to the Center for Disease Control (CDC), *C. difficile* is responsible for tens of thousands of cases of diarrhea and at least 5,000 deaths each year in the United States. The number of *C. difficile* infections doubled between 1993 and 2003, with the largest increase coming after 2000.

Individuals with a *C. difficile*-associated disease shed spores in the stool. *C. difficile* infections are frequently transmitted between hospitalized patients and the organism is often present on the hands of hospital personnel (see, for example, McFarland et al., 1989, *N Engl J Med*; 320:204-210). Patients infected with a *C. difficile* infection are isolated and precautions are taken to avoid outbreaks. Asymptomatic carriers can shed spores and need to be screened for isolation purposes (see, for example, Kyne et al., 2000, *N Engl J Med*; 342:390-397).

C. difficile spores are resistant to heat, drying, and cleaning agents and can survive up to seventy days on environmental surfaces, such as cart handles, bedrails, bedpans, toilets, bathing tubs, floors, furniture, linens, telephones, stethoscopes, thermometers, and remote controls. Thus, environmental surfaces are a ready source of infection. The thorough cleaning of patient's rooms during hospitalization is needed.

There is a clear need to monitor cleaning effectiveness and to verify that patient rooms and environmental surfaces are free of *C. difficile* spores. Currently, there are no easy to use, rapid methods for detecting *C. difficile* spores in environmental and patient samples. While kits (both immunoassay and molecular assays) are currently commercially available for the detection of *C. difficile* toxin, these kits do not detect *C.*

difficile spores. Thus, there is a need for rapid and easy to use systems for the detection of *C. difficile* spores.

SUMMARY OF THE INVENTION

The present invention includes an isolated antibody that binds to a *Clostridium difficile* spore. In some embodiments, the spore is an ungerminated spore. In some embodiments, the spore is a germinated spore. In some embodiments, the antibody does not bind to *Clostridium difficile* vegetative cells. In some embodiments, the antibody does not bind to *C. difficile* toxin.

The present invention includes an isolated antibody that binds to hypothetical protein CD1021 of *Clostridium difficile* strain 630 having SEQ ID NO: 1, or a fragment of hypothetical protein CD1021. In some embodiments, the isolated antibody binds a fragment of hypothetical protein CD1021 including amino acid residues 505 to 604. In some embodiments, the isolated antibody binds a fragment of hypothetical protein CD1021 including amino acid residues 30 to 120. In some embodiments, the isolated antibody binds a fragment of hypothetical protein CD1021 including amino acid residues 194 to 293. In some embodiments, the isolated antibody binds to a fragment of hypothetical protein CD1021 including amino acid residues 203 to 217. In some embodiments, the isolated antibody binds to a fragment of hypothetical protein CD1021 including amino acid residues 333 to 347.

The present invention includes an isolated antibody that binds to the amino acid sequence SEQ ID NO:2.

The present invention includes an isolated antibody that binds to the amino acid sequence SEQ ID NO:9.

The present invention includes an isolated antibody that binds to the amino acid sequence SEQ ID NO:10.

The present invention includes an isolated antibody that binds to the amino acid sequence EGSSLQYKGDDPESY (SEQ ID NO:3).

The present invention includes an isolated antibody that binds to the amino acid sequence LKNETYKTKYHKYLE (SEQ ID NO:4).

The present invention includes an isolated antibody that binds to putative N-acetylmuramoyl-L-alanine amidase protein of *Clostridium difficile* strain 630 having SEQ ID NO: 5 or a fragment of the putative N-acetylmuramoyl-L-alanine amidase

protein. In some embodiments, the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein including amino acid residues 294 to 393. In some embodiments, the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein including amino acid residues 582 to 596. In some embodiments, the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein including amino acid residues 64 to 78.

The present invention includes an isolated antibody that binds to the amino acid sequence SEQ ID NO:6.

The present invention includes an isolated antibody that binds to the amino acid sequence YKLKDKNGGTTKTVA (SEQ ID NO:7).

The present invention includes an isolated antibody that binds to the amino acid sequence KFKEKPDADSIKLKY (SEQ ID NO:8).

The present invention includes a monoclonal antibody, or antigen binding fragment thereof, wherein the monoclonal antibody or antigen binding fragment inhibits the binding of an antibody of the present invention to its antigen target.

The present invention includes an antigen binding fragment of an isolated antibody of the present invention.

In some embodiments, an isolated antibody of the present invention is a polyclonal antibody. In some embodiments, an isolated antibody of the present invention is a monoclonal antibody. In some embodiments, the isolated antibody of the present invention does not bind to *Bacillus subtilis* spores or *Clostridium sporogenes* spores. In some embodiments, the antibodies and antigen binding fragments of the present invention are labeled.

The present invention includes a composition including one or more of the isolated antibodies of the present invention, or antigen binding fragments thereof.

The present invention includes a kit including one or more of the isolated antibodies of the present invention, or antigen binding fragments thereof.

The present invention includes a hybridoma cell line or transformed B cell line that produces a monoclonal antibody of the present invention.

The present invention includes an isolated polynucleotide sequence including the nucleic acid sequence coding for the heavy chain, the light chain, the heavy chain variable region, the light chain variable region, or one or more complementarity

determining regions of a monoclonal antibody of the present invention. The present invention includes an expression vector including such an isolated polynucleotide sequence. The present invention includes a host cell including such an expression vector.

The present invention includes a method of preparing an anti-*Clostridium difficile* antibody, the method including immunizing a host organism with a polypeptide including at least a portion of a protein encoded by the *C. difficile* genome in an amount effective to generate an antibody response to the polypeptide. In some embodiments, the polypeptide including at least a portion of a protein encoded by the *C. difficile* genome is selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof. In some embodiments, the method further includes purifying the antibody preparation.

The present invention includes a method of preparing an anti-*Clostridium difficile* antibody, the method including expressing a nucleic acid sequence encoding at least a portion of a protein encoded by the *C. difficile* genome in an immunocompetent host organism. In some embodiments, the nucleic acid sequence encoding at least a portion of a protein encoded by the *C. difficile* genome encodes an amino acid sequence selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof. In some embodiments, the method further includes purifying the antibody preparation.

The present invention includes a composition including at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *Clostridium difficile* spore.

In some embodiments of the composition, at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

In some embodiments of the composition, at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine

amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

In some embodiments of the composition, a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof. In some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

The present invention includes a kit including at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *Clostridium difficile* spore.

In some embodiments of the kit, at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

In some embodiments of the kit, at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

In some embodiments of the kit, a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having

SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof. In some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

The present invention includes a method of detecting the presence of a *Clostridium difficile* spore in a sample, the method including contacting the sample with one or more isolated antibodies of the present invention.

The present invention includes a method of detecting the presence of a *Clostridium difficile* spore in a sample, the method including contacting the sample with at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *C. difficile* spore.

The present invention includes a method of detecting the presence of a *Clostridium difficile* spore in a sample, the method including: contacting the sample with a first isolated antibody or antigen-binding fragment thereof, wherein the first isolated antibody binds to a first antigenic epitope of the *C. difficile* spore; and contacting the sample with a second isolated antibody or antigen-binding fragment thereof, wherein the second isolated antibody binds to a second antigenic epitope of the *C. difficile* spore.

In some embodiments of the methods of the present invention, at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

In some embodiments of the methods of the present invention, at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In

some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

In some embodiments of the methods of the present invention, a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein. In some embodiments, the polypeptide fragment of hypothetical protein CD1021 is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof. In some embodiments, the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents the homology between the amino acid sequences for hypothetical CD1021 proteins from *C. difficile* strain 630 (SEQ ID NO:1, corresponding to GenBank Accession No. YP_001087502), *C. difficile* QCD-32g58 (SEQ ID NO:11, corresponding to GenBank Accession No. ZP_01804840), *C. difficile* QCD-32g58 (SEQ ID NO:12, corresponding to GenBank Accession No. ZP_01804841), *C. difficile* QCD-32g58 (SEQ ID NO:21, translated from the region corresponding to nucleotides 461827 to 462825 of GenBank Accession No. NZ_AAML04000007), *C. difficile* QCD-32g58 (SEQ ID NO:22, translated from region corresponding to nucleotides 462824 to 463732 of GenBank Accession No. NZ_AAML04000007), and from *C. difficile* QCD-66c26 (SEQ ID NO:23, translated from the complement of the region corresponding to nucleotides 15690 to 17597 of GenBank Accession No. NZ_ABFD01000037). The sequences were aligned using the multiple sequence alignment program CustalW, which is publicly available at http://www.ebi.ac.uk/Tools/custalw/. The consensus sequence as shown is SEQ ID NO:38. Amino acid residues that are identical in at least four of the six hypothetical

proteins are shown in the consensus sequence. An "X" residue in the consensus sequence indicates that two or more of the aligned sequences showed nonidentity at the respective residue or it indicates that sequence information was lacking for the respective residue in three or more of the aligned sequences. A "-" symbol located at any given position in one of the aligned sequences indicates that amino acid position was unreported in the corresponding GenBank entry.

DETAILED DESCRIPTION

The present invention relates to antibodies that bind to the endospore of the bacterium *Clostridium difficile* (also referred to herein as "*C. difficile*," "C. diff," "c. diff," "C-diff," or "C.D"). Such spore-specific antibodies are useful, for example, in the detection of *C. difficile* endospores in environmental, biological, and food samples. Only a few genera of bacteria, such as, for example, *Bacillus* and *Clostridium*, are capable of forming endospores. Bacterial endospores are highly resistant to hostile physical and chemical conditions, proving to be one of the most durable types of cells found in nature. They can survive high heat, drying, radiation, and many damaging chemicals and are a dormant form of the bacterium that allows it to survive sub-optimal environmental conditions. Endospores can survive for a very long time and then return to a growing state, a process termed germination. Because endospores are resistant to heat, radiation, disinfectants, and desiccation, they are difficult to eliminate from medical and pharmaceutical materials and are a frequent cause of contamination.

Antibodies of the present invention bind to the endospore (also referred to herein as "spore") of the bacterium *C. difficile*. As used herein, the terms "antibody" or "antibodies" are used interchangeably. An antibody of the present invention may bind to both viable spores and inactivated *C. difficile* spores. Spores may be inactivated by any of a variety of methods, including, but not limited to, for example, treatment with formalin, formaldehyde, glutaraldehydes, chemical disinfectants, autoclaving, and ultraviolet radiation. Antibodies of the present invention may bind to both germinated and ungerminated *C. difficile* spores. Antibodies of the present invention may bind to ungerminated *C. difficile* spores and not bind to germinated *C. difficile* spores. Antibodies of the present invention may bind to germinated *C. difficile* spores and not bind to ungerminated *C. difficile* spores. Methods for the preparation of ungerminated spores and germinated spores are well known to the skilled artisan. Briefly, bacterial

spores are generally prepared by growing the bacteria on media such as tryptic soy agar or in tryptic soy broth until most cells turn into spores. Spores are collected by centrifugation and washed several times with a buffer such as PBS. The suspension can be treated with alcohol to kill vegetative cells and washed to collect spores (see, for example, Long and Williams, 1958, *J Bacteriol*: 76:332 and Powers, 1968, *Appl Microbiol*; 16:180-181). Spore germination can be triggered by a variety of methods. See, for example, Gould, 1970, *J Appl Bacteriol*; 33:34-49; Foerster and Foster, 1966, *J Bacteriol*; 91:1168-1177; Moir and Smith, 1990, *Ann Rev Microbiol*; 44:531-553; and U.S. Patent Application Serial No. 2003/0175318A1.

Antibodies of the present invention may bind *C. difficile* spores and not bind to the spores of other endospore-forming bacteria. Antibodies of the present invention may bind *C. difficile* spores and not bind to the spores of other endospore-forming bacteria of the *Firmicute* phylum, such as for example, endospores produced by any of the various species of the *Clostridium* or *Bacillus* genera. Species of the *Clostridium* and *Bacillus* genera of bacteria include, but are not limited to, *Clostridium aceticum*, *Clostridium acetobutylicum*, *Clostridium botulinum*, *Clostridium butyricum*, *Clostridium carnis*, *Clostridium chauvoei*, *Clostridium denitrificans*, *Clostridium fervidus*, *Clostridium formicoaceticum*, *Clostridium novyi*, *Clostridium pasteurianum*, *Clostridium perfringens*, *Clostridium septicum*, *Clostridium sporogenes*, *Clostridium tetani*, *Clostridium thermoaceticum*, *Clostridium thermocellum*, *Clostridium thermosacchrolyticum*, *Clostridium tyrobutyricum*, *Clostridium welchii*, *Bacillus agaradhaerens*, *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus anthracis*, *Bacillus atrophaeus*, *Bacillus azotoformans*, *Bacillus badius*, *Bacillus benzoevorans*, *Bacillus carboniphilus*, *Bacillus cereus*, *Bacillus chitinolyticus*, *Bacillus circulans*, *Bacillus clarkii*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus cohnii*, *Bacillus edaphicus*, *Bacillus ehimensis*, *Bacillus fastidiosus*, *Bacillus firmus*, *Bacillus flexus*, *Bacillus fumarioli*, *Bacillus fusiformis*, *Bacillus gibsonii*, *Bacillus globisporus*, *Bacillus halmapalus*, *Bacillus haloalkaliphilus*, *Bacillus halodenitrificans*, *Bacillus halodurans*, *Bacillus halophilus*, *Bacillus horikoshii*, *Bacillus horti*, *Bacillus infernos*, *Bacillus insolitus*, *Bacillus kaustophilus*, *Bacillus laevolacticus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus marinus*, *Bacillus megaterium*, *Bacillus methanolicus*, *Bacillus mojavensis*, *Bacillus mucilaginosus*, *Bacillus mycoides*, *Bacillus naganoensis*, *Bacillus niacini*, *Bacillus oleronius*, *Bacillus pallidus*, *Bacillus pasteurii*, *Bacillus*

pseudalcaliphilus, *Bacillus Pseudofirmus*, *Bacillus pseudomycoides*, *Bacillus psychrophilus*, *Bacillus psychrosaccharolyticus*, *Bacillus pumilus*, *Bacillus schlegelii*, *Bacillus silvestris*, *Bacillus simplex*, *Bacillus siralis*, *Bacillus smithii*, *Bacillus sphaericus*, *Bacillus sporothermodurans*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thermoamylovorans*, *Bacillus thermocatenulatus*, *Bacillus thermocloaceae*, *Bacillus thermodenitrificans*, *Bacillus thermoglucosidasius*, *Bacillus thermoleovorans*, *Bacillus thermosphaericus*, *Bacillus thuringiensis*, *Bacillus tusciae*, *Bacillus vallismortis*, *Bacillus vedderi*, *Bacillus vulcani*, and *Bacillus weihenstephanensis*.

Antibodies of the present invention may bind *C. difficile* spores and not bind to the spores of other bacteria, such as, for example, *Desulfotomaculum*, *Sporolactobacillus*, *Brevibacillus*, *Sporosarcina*, and *Thermoactinomyces*.

In some embodiments, antibodies of the present invention bind to *C. difficile* spores and do not bind to the spores of *Bacillus subtilis* (also referred to herein as *B. subtilis*) and *Clostridium sporogenes* (also referred to herein as *C. sporogenes*).

Antibodies of the present invention may bind to *C. difficile* spores and not bind to *C. difficile* vegetative cells. Antibodies of the present invention may bind to *C. difficile* spores and not bind to vegetative cells of other endospore-forming bacteria, including any of those described herein. In some embodiments, antibodies of the present invention do not bind to vegetative cells of *C. difficile*, *B. subtilis*, and *C. sporogenes*. Methods for culturing vegetative cells of a wide variety of *Clostridium* and *Bacillus* species, including, but not limited *C. difficile*, *C. sporogenes*, and *B. subtilis*, are well known to the skilled artisan. See, for example, Madigan et al., 2003, Brock Biology of Microorganisms, Prentice Hall; and Cappucino, 2005, Microbiology Laboratory Manual, Benjamin Cummings.

Pathogenic *C. difficile* strains produce various toxins. The best characterized are enterotoxin (toxin A) and cytotoxin (toxin B) and these two toxins are responsible for the diarrhea and inflammation seen in infected patients (see, for example, Gianfrilli et al., 1984, *Microbiologica*; 7:375-9). Antibodies of the present invention may bind to *C. difficile* spores and not bind to a toxin produced by *C. difficile*, for example, the antibody may not bind to toxin A and/or toxin B. Methods for preparing *C. difficile* toxin A and toxin B and determining if an antibody binds to *C. difficile* toxin A and/or toxin B are well known to the skilled artisan. See, for example, U.S. Patent Nos.

4,530,833; 4,533,630; 4,863,852; 4,879,218; 5,231,003; 5,610,023; 5,965,375; 6,503,722; 6,939,548; and 7,179,611.

Bacterial endospores, including *C. difficile* endospores, are encased in a multilayered protein structure formed by the ordered assembly of many polypeptides. The endospore contains four protective layers, the core, the cortex, the coat, and the exosporium. The outermost layer of the spore is the exosporium, a thin covering made of protein. Interior to this is the spore coat which is made up of highly cross-linked keratin and layers of spore-specific proteins. The spore coat is impermeable to many toxic molecules and may also contain enzymes that are involved in germination. The cortex lies beneath the spore coat and consists of peptidoglycan. The core wall lies beneath the cortex and surrounds the protoplast or core of the endospore. The core has normal cell structures, such as DNA and ribosomes, but is metabolically inactive.

Some embodiments of the present invention include antibodies that bind to a spore-specific protein, such as, for example, an exosporium protein, a spore coat protein, a spore cortex protein, a spore inner membrane protein, or a spore core protein of *C. difficile*. Such an antibody may bind to a spore-specific protein found on one or more endospore-forming bacteria of the *Firmicute* phylum described herein. In some embodiments, the antibody binds to a spore-specific protein found in *C. difficile,* but does not bind to the spore-specific protein other endospore-forming bacteria of the *Firmicute* phylum, such as, for example, *B. subtilis* and *C. sporogenes*.

Some embodiments of the present invention include antibodies that bind to a spore coat assembly protein of *C. difficile*. One such spore coat assembly protein is the CotH protein (also referred to herein as "cotH"). The CotH protein is a structural component of the spore coat and has been well characterized in *B. subtilis*. It is involved in directing the assembly of coat proteins and in stabilizing coat proteins. See, for example, Naclerio et al., 1996, *J Bacteriol*; 178(15):4375–4380 and Zilha et al., 1999, *J Bacteriol*; 181:2631–2633). The present invention includes antibodies that bind to a putative CotH protein in *C. difficile*.

The complete genome sequence of *C. difficile* strain 630 has been determined and is available in the GenBank® sequence database maintained by the National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), National Institutes of Health (NIH). See also, Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786). Strain 630 is multi-drug resistant and was isolated from a patient with severe

pseudomembraneous colitis that had spread to dozens of other patients on the same ward in Zurich, Switzerland in 1982 (Wren, 2006, *Future Microbiol*; 1(3):243-245). Thus, strain 630 has the genetic attributes of a fully virulent, highly transmissible, drug resistant strain.

Efforts are currently under way to obtain the complete genome sequences of other *C. difficile* strains. The Sanger Institute (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) is sequencing the genome of *C. difficile* strain R20291. *C. difficile* strain R20291 was isolated in Stoke Mandeville, UK, and is closely related to the North American hypervirulent BI strains (see the ftp site sanger.ac.uk/pub/pathogens/cd/C_difficile_Bi_454.dbs). Washington University in St. Louis (St. Louis, MO) is sequencing the genome of *C. difficile* QCD-32g58 (see the worldwide web at cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntcd03).

A thorough search of all the GenBank® entries for *C. difficile* strain 630 identified hypothetical protein CD1021 (YP_001087502), which demonstrates a conserved domain (amino acid residues 90 to 393) which is homologous to the spore coat assembly protein H (cotH) of *B. subtilis*. The analysis was performed using conserved domain search tools available from the NCBI and available on the worldwide web at ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. Hypothetical Protein CD1021 of *C. difficile 630* (GenBank Accession No. YP_001087502) has the amino acid sequence SEQ ID NO:1. See Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786 and the worldwide web at ncbi.nlm.nih.gov/entrez/ viewer.fcgi?db=protein&id=126698605.

Some embodiments of the present invention include antibodies that bind to the hypothetical protein CD1021 of *C. difficile*, and fragments thereof. An antibody of the present invention may bind to a hypothetical protein CD1021 in a variety of *C. difficile* strains, including, but not limited to, any of the *C. difficile* strains discussed herein. For example, an antibody of the present invention may bind to the hypothetical protein CD1021 of *C. difficile* strain 630, *C. difficile* strain R20291, *C. difficile* strain QCD-32q58, *C. difficile* strain QCD-66c26, *C. difficile* ATCC 43255, *C. difficile* ATCC 43593, *C. difficile* ATCC 43594, *C. difficile* ATCC 43596, *C. difficile* ATCC 43597, *C. difficile* ATCC 43598, *C. difficile* ATCC 43603, *C. difficile* ATCC 9689, and/or *C. difficile* ATCC 700792. Antibodies of the present invention include antibodies that bind to the hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO:1.

Some embodiments of the present invention include antibodies that bind to polypeptide fragments of the hypothetical protein CD1021 of *C. difficile*. A polypeptide fragment may be, for example, about 50, about 100, about 200, about 300, about 400, about 500, or about 600 amino acids in length. A polypeptide fragment may be, for example, about 10, about 15, about 20, about 25, about 30, about 35, about 40, or about 45 amino acids in length. A polypeptide fragment may be about 8-20, about 12-15, or about 10-20 amino acids in length.

Some embodiments of the present invention include antibodies that bind to polypeptide fragments of the hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:1). For example, the present invention includes antibodies that bind to a polypeptide including residues 505-604 of hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:2), antibodies that bind to a polypeptide including residues 30-120 of hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:9), antibodies that bind to a polypeptide including residues 194-293 of hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:10), antibodies that bind to a polypeptide including residues 203 to 217 of hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:3), and antibodies that bind to a polypeptide including residues 333 to 347 of hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:4).

The spore cortex, a thick layer of peptidoglycan, is responsible for maintaining the highly dehydrated state of the spore and contributes to the extreme dormancy and heat resistance of the spores. Bacterial spore germination includes a series of degradation events that lead to the irreversible loss of spore dormancy and the rehydration of the core. The spore contains enzymes that are involved in germination. Thus, an antibody that binds to a spore-specific protein involved in germination may be used to identify germinating spores. The present invention includes antibodies that bind to a spore-specific protein involved in germination. Such an antibody may bind to germinated spores but not bind to ungerminated spores. Such an antibody may bind to ungerminated spores but not bind to germinated spores.

Cortex lytic enzymes, including the amidase N-acetylmuramoyl L-alanine amidase, play a key role in germination, resulting in hydrolysis of the cortex (see, for example, Moriyama et al., 1996, *J Bacteriol*; 181:2373–2378). The present invention

includes antibodies that bind to a *C. difficile* amidase, including antibodies that bind to the N-acetylmuramoyl-L-alanine amidase of *C. difficile*.

A thorough search of all the GenBank® entries for *C. difficile* strain 630 identified cell surface protein (putative N-acetylmuramoyl-L-alanine amidase, YP_001087517, also referred to herein as "CD1036") which has conserved domains CW_binding_2 (putative cell wall binding repeat 2; 174 to 265, 275 to 368, 381 to 461) and Amidase_3 (N-acetylmuramoyl-L-alanine amidase, 493 to 673). The putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* 630 (GenBank Accession No. YP_001087517) has the amino acid sequence SEQ ID NO:5. See Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786 and the worldwide web at ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id.

The present invention includes antibodies that bind to the putative N-acetylmuramoyl-L-alanine amidase of *C. difficile*, and fragments thereof. An antibody of the present invention may bind to the putative N-acetylmuramoyl-L-alanine amidase in a variety of *C. difficile* strains, including, but not limited to, any of the *C. difficile* strains discussed herein. For example, an antibody of the present invention may bind to the putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630, *C. difficile* strain R20291, *C. difficile* strain QCD-32q58, *C. difficile* ATCC 43255, *C. difficile* ATCC 43593, *C. difficile* ATCC 43594, *C. difficile* ATCC 43596, *C. difficile* ATCC 43597, *C. difficile* ATCC 43598, *C. difficile* ATCC 9689, and/or *C. difficile* ATCC 700792. Antibodies of the present invention include antibodies that bind to the putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630 having SEQ ID NO:5.

Some embodiments of the present invention include antibodies that bind to polypeptide fragments of the putative N-acetylmuramoyl-L-alanine amidase. A polypeptide fragment may be, for example, about 50, about 100, about 200, about 300, about 400, about 500, or about 600 amino acids in length. A polypeptide fragment may be, for example, about 10, about 15, about 20, about 25, about 30, about 35, about 40, or about 45 amino acids in length. A polypeptide fragment may be about 8-20, about 12-15, or about 10-20 amino acids in length. The present invention includes antibodies that bind to polypeptide fragments of the putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630 having SEQ ID NO:5. For example, the present invention includes antibodies that bind to a polypeptide including residues 294-393 of putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630 (SEQ ID

NO:6), antibodies that bind to a polypeptide including residues 582-596 of putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630 (SEQ ID NO:7), and antibodies that bind to a polypeptide including residues 64-78 of putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* strain 630 (SEQ ID NO:8).

Antibodies of the present invention include, but are not limited to, polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, anti-idiotypic antibodies, multispecific antibodies, single chain antibodies, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, F(ab')2 fragments, Fv fragments, diabodies, linear antibodies fragments produced by a Fab expression library, fragments comprising either a VL or VH domain, intracellularly-made antibodies (i.e., intrabodies), and antigen-binding antibody fragments thereof. Any of a wide variety of target antigens may be used to produce the antibodies of the present invention, including, but not limited to, *C. difficile* cells, spores or toxins, proteins, peptides, carbohydrates and combinations thereof. Proteins and peptides may be, for example, naturally occurring, chemically synthesized, or recombinantly produced. An antigen may be conjugated to a carrier.

Also included in the present invention are various antibody fragments, also referred to as antigen binding fragments, which include only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Examples of antibody fragments include, for example, Fab, Fab', Fd, Fd', Fv, dAB, and F(ab')2 fragments produced by proteolytic digestion and/or reducing disulfide bridges and fragments produced from an Fab expression library. Such antibody fragments can be generated by techniques well known in the art. Antibodies of the present invention can include the variable region(s) alone or in combination with the entirety or a portion of the hinge region, CH1 domain, CH2 domain, CH3 domain and/or Fc domain(s). The term "antigen-binding fragment" refers to a polypeptide fragment of an immunoglobulin or antibody that binds antigen or competes with the intact antibody for antigen binding.

The antibodies of the present invention can be of any type (such as, for example, IgG, IgE, IgM, IgD, IgA and IgY), class (such as, for example, IgG1, IgG2,

IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. In some embodiments, the immunoglobulin is an IgG. Immunoglobulins can have both heavy and light chains. An array of IgG, IgE, IgM, IgD, IgA, and IgY heavy chains can be paired with a light chain of the kappa or lambda form.

The antibodies of the invention can be from any animal origin, including birds and mammals. In some embodiments, the antibodies are human, murine, rat, donkey, sheep, rabbit, goat, guinea pig, camel, horse, llama, camel, or chicken antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins.

Antibodies of the present invention may be a polyclonal antibody. The term "polyclonal antibody" refers to an antibody produced from more than a single clone of plasma cells. In contrast "monoclonal antibody" refers to an antibody produced from a single clone of plasma cells. The preparation of polyclonal antibodies is well known.

A polyclonal antibody to a target antigen may be obtained by immunizing any of a variety of host animals with an immunogen. Any of a wide variety of immunization protocols may be used. The host animal may be any mammal, for example, a mouse, hamster, rat, rabbit, guinea pig, goat, sheep, horse, cow, buffalo, bison, camel, or llama. A host animal may be a bird, for example, a chicken or a turkey. In some embodiments, an antibody preparation, rather than obtained from a blood sample, is obtained from another fluid source, for example, from milk, colostrums, egg white, or egg yolk. In some embodiments, an antibody preparation is obtained, not by immunizing a host animal with the target antigen, but rather, from an individual with a prior exposure to the antigen or from pooled serum, for example, from pooled human serum.

It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include, but are not limited to, keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

Some embodiments of the present invention include antiserum that binds to a *C. difficile* spore. As used herein, antiserum refers to the blood from an immunized host animal from which the clotting proteins and red blood cells (RBCs) have been removed. An antiserum (also referred to herein as an "antiserum preparation," "crude antiserum," or "raw antiserum") still possesses immunoglobulins of all classes as well as other various serum proteins. Thus, in addition to antibodies that recognize the target antigen, the antiserum also contains antibodies to various non-target antigens that can sometimes react non-specifically in immunological assays.

In some embodiments of the present invention, an antibody may be enriched. Such enrichment may eliminate non-immunoglobulin proteins from the preparation and/or enrich for one or more classes of immunoglobulin (such as, for example, IgG) within the sample. Any of a variety of methods may be used to obtain such an enriched antibody, including, but not limited to, those described herein. Methods of eliminating non-immunoglobulin serum proteins from an antibody preparation and methods for enriching for the IgG fraction are well known in the art. For example, ammonium sulfate precipitation, Protein A binding, Protein G binding, or caprylic acid precipitation may be used to enrich for the IgG class of antibodies.

Antibodies of the present invention include antibodies with enhanced avidity for the target antigen. Such antibodies may be prepared by antigen affinity immunoadsorption. Antigen affinity immunoadsorption may be carried out by any of a variety of means. For example, antigen affinity immunoadsorption may be carried out by antigen affinity column chromatography. Column chromatography may be carried out by any mechanical means, for example, carried out in a column run with or without pressure, carried out in a column run from top to bottom or bottom to top, or the direction of the flow of fluid in the column may be reversed during the chromatography process. Alternatively, antigen affinity immunoadsorption may be carried out by means other than column chromatography. For example, affinity immunoadsorption may be carried out using a batch process in which the solid support is separated from the liquid used to load, wash, and elute the sample by any suitable means, including gravity, centrifugation, or filtration. Affinity immunoadsorption also may be carried out by contacting the sample with a filter that adsorbs or retains some molecules in the sample more strongly than others. The antigen affinity column may be prepared by any of a variety of methods, including, but not limited to, those described herein. The

binding of an antibody preparation to an antigen affinity column may be carried out by any of a wide variety of immunoadsorption methods, including, but not limited to, those described herein. The binding of the antibody preparation to the antigen affinity column may occur in a variety of buffers or salts including, but not limited to, sodium, potassium, ammonium, chloride, acetate, phosphate, citrate, Tris buffers and/or organic buffers with a buffering capacity near neutrality. Specific examples of such buffers and salts include, for example, Tris, sodium phosphate, potassium phosphate, ammonium phosphate, sodium chloride, potassium chloride, ammonium chloride, sodium citrate, potassium citrate, ammonium citrate, sodium acetate, potassium acetate, or ammonium acetate.

Antibodies of the present invention include monoclonal antibodies. A population of monoclonal antibodies is homogeneous. All of the monoclonal antibodies in the preparation recognize the same epitope on the target molecule and all of the monoclonal antibodies have the same affinity. As used herein, "affinity" is the binding strength of the interaction of a monoclonal antibody with its antigenic epitope. As used herein, an "epitope" is the portion of an antigen bound by an antibody. The higher the affinity, the tighter the association between antigen and antibody, and the more likely the antigen is to remain in the binding site.

Monoclonal antibodies of the present invention include, but are not limited to, humanized antibodies, chimeric antibodies, single chain antibodies, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, F(ab')2 fragments, Fv fragments, diabodies, linear antibodies fragments produced by a Fab expression library, fragments including either a VL or VH domain, intracellularly-made antibodies (i.e., intrabodies), and antigen-binding antibody fragments thereof.

Monoclonal antibodies of the present invention can be produced by an animal (including, but not limited to, human, mouse, rat, rabbit, hamster, goat, horse, chicken, or turkey), chemically synthesized, or recombinantly expressed. Monoclonal antibodies of the present invention can be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to

heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

Monoclonal antibodies of the present invention may be of any isotype. The monoclonal antibodies of the present invention may be, for example, murine IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, IgD, or IgE. The monoclonal antibodies of the present invention may be, for example, human IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, or IgE. In some embodiments, the monoclonal antibody may be murine IgG2a, IgG1, or IgG3. With the present invention, a given heavy chain may be paired with a light chain of either the kappa or the lambda form.

Monoclonal antibodies can be obtained by various techniques familiar to those skilled in the art. For example, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, for example, Kohler and Milstein, 1976, *Eur J Immunol*; 6:511-519; J. Goding In "Monoclonal Antibodies: Principles and Practice," Academic Press, pp 59-103 (1986); and Harlow et al., Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. (1988)). Monoclonal antibodies can be isolated and purified from hybridoma cultures by techniques well known in the art. Other known methods of producing transformed B cell lines that produce monoclonal antibodies may also be used. Monoclonal antibodies of the present invention may be produced by recombinant DNA techniques, for example, produced by phage display or by combinatorial methods. See, for example, U.S. Patent No. 5,223,409; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; or WO 90/02809. Such methods can be used to generate human monoclonal antibodies.

Monoclonal antibodies of the present invention include chimeric antibodies. A chimeric antibody is one in which different portions are derived from different animal species. For example, chimeric antibodies can be obtained by splicing the genes from a mouse antibody molecule with appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological specificity. See, for example, Takeda et al., 1985, *Nature*; 314:544-546.

A therapeutically useful antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring one or more CDRs from the heavy and light variable chains of a mouse (or other species) immunoglobulin into a human variable domain, then substituting human

residues into the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with immunogenicity of murine constant regions. Techniques for producing humanized monoclonal antibodies can be found, for example, in Jones et al., 1986, *Nature*; 321:522 and Singer et al., 1993, *J Immunol*: 150:2844. The constant region of a humanized monoclonal antibody of the present invention can be that from human immunoglobulin belonging to any isotype. It may be, for example, the constant region of human IgG. The framework regions of the constant region derived from human immunoglobulin are not particularly limited.

An intact antibody molecule has two heavy (H) chain variable regions (abbreviated herein as VH) and two light (L) chain variable regions (abbreviated herein as VL). The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al., *J. Mol. Biol.* 1987;196: 901-917). Each VH and VL is composed of three CDR's and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The present invention includes an antibody with the heavy chain, the light chain, the heavy chain variable region, the light chain variable region, and/or one or more complementarity determining regions of a monoclonal antibody of the present invention.

The present invention includes bispecific or bifunctional antibodies. A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of F(ab') fragments. See, for example, Songsivilai and Lachmann, 1990, *Clin Exp Immunol*; 79:315-321 and Kostelny et al., 1992, *J Immunol*; 148:1547-1553. In addition, bispecific antibodies can be formed as "diabodies" (Holliger et al., 1993, *PNAS USA*: 90:6444-6448) or "Janusins" (Traunecker et al., 1991, *EMBO J*; 10:3655-3659 and Traunecker et al., 1992, *Int J Cancer Suppl*; 7:51-52).

Also included in the present invention are hybridoma cell lines, transformed B cell lines, and host cells that produce the monoclonal antibodies of the present invention; the progeny or derivatives of these hybridomas, transformed B cell lines, and host cells; and equivalent or similar hybridomas, transformed B cell lines, and host cells. Progeny or derivatives thereof may produce an antibody with one or more of the identifying characteristics, such as, for example, isotype and antigen specificity, of the antibody produced by the parental line.

The present invention further provides an isolated polynucleotide molecule having a nucleotide sequence encoding a monoclonal antibody of the invention. The present invention is further directed to an isolated polynucleotide molecule having a nucleotide sequence that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to nucleotide sequence encoding a monoclonal antibody of the invention. The invention also encompasses polynucleotides that hybridize under high stringency to a nucleotide sequence encoding an antibody of the invention, or a complement thereof. As used herein "stringent conditions" refer to the ability of a first polynucleotide molecule to hybridize, and remain bound to, a second, filter-bound polynucleotide molecule in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 65° C, followed by washing in 0.2 X SSC/0.1% SDS at 42° C (see Ausubel et al. (eds.), Current Protocols in Molecular Biology, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., NY, at p. 2.10.3 (1989)). Also included in the present invention are polynucleotides that encode one or more of the CDR regions or the heavy and/or light chains of a monoclonal antibody of the present invention. General techniques for cloning and sequencing immunoglobulin variable domains and constant regions are well known. See, for example, Orlandi et al., 1989, *PNAS USA*; 86:3833.

The present invention also includes recombinant vectors including an isolated polynucleotide of the present invention. The vector can be, for example, in the form of a plasmid, a viral particle, or a phage. The appropriate DNA sequence can be inserted into a vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) in a vector by procedures known in the art. Such procedures are deemed to be within the scope of those skilled in the art. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of

example. Bacterial vectors include, for example, pQE70, pQE60, pQE-9, pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, and pRIT5. Eukaryotic vectors include, for example, pWLNEO, pSV2CAT, pOG44, pXT1, pSG, pSVK3, pBPV, pMSG, and pSVL. However, any other plasmid or vector can be used.

Some embodiments of the present invention also include host cells containing the above-described vectors. The host cell can be a higher eukaryotic cell, such as a mammalian or insect cell, or a lower eukaryotic cell, such as a yeast cell. Or, the host cell can be a prokaryotic cell, such as a bacterial cell, or a plant cell. Introduction of a vector construct into the host cell can be effected by any suitable techniques, such as, for example, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., et al., Basic Methods in Molecular Biology (1986)).

Monoclonal antibodies of the present invention can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989).

Also included in the present invention are phage display libraries expressing one or more hypervariable regions from a monoclonal antibody of the present invention, and clones obtained from such a phage display library. A phage display library is used to produce antibody derived molecules. Gene segments encoding the antigen-binding variable domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophage. Phage display libraries can be prepared, for example, using the Ph.D.™-7 Phage Display Peptide Library Kit (Catalog No. E8100S) or the Ph.D.™-12 Phage Display Peptide Library Kit (Catalog No. E8110S) available from New England Biolabs Inc., Ipswich, MA. See also, Smith and Petrenko, 1997, *Chem Rev*; 97:391-410.

The antibodies of the present invention may be coupled directly or indirectly to a substrate or detectable marker by techniques well known in the art. A detectable marker is an agent detectable, for example, by spectroscopic (e.g., u.v., i.r., visible, Raman, surface enhanced Raman scattering (SERS), mass spectroscopy), photochemical, biochemical, immunochemical, or chemical means. Useful detectable markers include, but are not limited to, fluorescent dyes, chemiluminescent compounds, radioisotopes, electron-dense reagents, enzymes, colored particles, biotin, or dioxigenin. A detectable marker often generates a measurable signal, such as radioactivity, fluorescent light, color, or enzyme activity. Antibodies conjugated to detectable agents may be used for diagnostic or therapeutic purposes. Examples of detectable agents include various enzymes, naturally-occurring spore-associated biomolecules (e.g., dipicolinic acid, calcium dipicolinate), prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, nonradioactive paramagnetic metal ions, Raman labels and SERS labels. The detectable substance can be coupled or conjugated either directly to the antibody or indirectly, through an intermediate such as, for example, a linker known in the art, using techniques known in the art. See, for example, U.S. Patent No. 4,741,900, describing the conjugation of metal ions to antibodies for diagnostic use. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, and acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferin, and aequorin; and examples of suitable radioactive material include iodine (^{121}I, ^{123}I, ^{125}I, ^{131}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{111}In, ^{112}In, ^{113}mIn, ^{115}mIn), technetium (^{99}Tc, ^{99}mTc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc, ^{186}Re, ^{188}Re, ^{142}Pr, ^{105}Rh, and ^{97}Ru. Techniques for conjugating such moieties to antibodies are well-known.

Antibodies of the present invention include derivatives of antibodies that are modified or conjugated by the covalent attachment of any type of molecule to the

antibody. Such antibody derivatives include, for example, antibodies that have been modified by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or linkage to a cellular ligand or other protein. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, and metabolic synthesis of tunicamycin. Additionally, the derivatives can contain one or more non-classical amino acids.

Antibodies of the present invention can be assayed for immunospecific binding by the methods described herein and by any suitable method known in the art. The immunoassays that can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as BIAcore analysis, fluorescence activated cell sorter (FACS) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art (see, for example, Ausubel et al., eds, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., NY (1994)).

Also included in the present invention are compositions including one or more of the antibodies described herein. A composition may also include, for example, buffering agents to help to maintain the pH in an acceptable range or preservatives to retard microbial growth. A composition may include, for example, carriers, excipients, stabilizers, chelators, salts, or antimicrobial agents. Acceptable carriers, excipients, stabilizers, chelators, salts, preservatives, buffering agents, or antimicrobial agents, include, but are not limited to, buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives, such as sodium azide, octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol; polypeptides; proteins, such as serum albumin, gelatin, or non-specific immunoglobulins; hydrophilic polymers such as olyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine;

monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (for example, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, PLURONICS, or polyethylene glycol (PEG). As used herein, a composition is not a polyclonal antiserum.

The invention also provides a kits or detection systems including one or more antibodies of the present invention. The kit may include one or more containers filled with one or more of the antibodies of the invention. Additionally, the kit may include other reagents such as buffers and solutions needed to practice the invention are also included. Optionally associated with such container(s) can be a notice or printed instructions. A kit can include packaging material. As used herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods, which can provide a sterile, contaminant-free environment.

The present invention includes isolated antibodies. "Isolated," when used to describe the various antibodies disclosed herein, means the antibody that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes.

The antibodies of the present invention may "specifically bind to" or be "specific for" a particular polypeptide or an epitope on a particular polypeptide. Such an antibody is one that binds to that particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

Antibodies of the present invention can be produced by an animal, chemically synthesized, or recombinantly expressed. Antibodies of the present invention can be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (including, but not limited to, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to heterologous

polypeptide sequences described herein or otherwise known in the art, to facilitate purification or detection.

The antibodies of the present invention may be used in a wide variety of diagnostic and therapeutic methods, including, but not limited to, methods for detecting *C. difficile* spores, and polypeptide fragments thereof, and methods for isolating or purifying *C. difficile* spores, or polypeptide fragments thereof.

The antibodies of the present invention may be used in any of the wide variety of immunoassay techniques known in the art to determine the presence or absence of *C. difficile* spores in a sample. As used herein, an immunoassay is a test that identifies the presence of an analyte, such as *C. difficile* spores, in a sample, using the reaction of an antibody to its antigen target. The assay takes advantage of the specific binding of an antibody to its antigen. Also included in the present invention are such methods of detection.

In an immunoassay, a sample is contacted with one or more antibodies and the antibodies are allowed to bind to their antigenic target, if present in the sample. Then, the binding of the one or more antibodies to their antigenic targets is determined, by detecting antibody bound to its antigen target. Such detection may be accomplished, for example, colorimetrically, fluorimetrically, enzymatically, or with radioactive isotopes. Depending on the format of the assay, detectable labels can be bound to an antigen or an antibody. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as, for example, ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as, for example, fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as, for example, alkaline phosphatase, beta-galactosidase or horseradish peroxidase, a Raman label, or a SERS label. Any method known in the art for conjugating an antibody or antigen to a detectable moiety may be employed. A detectable moiety (also referred to herein as a detectable label) may be conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

An immunoassay of the present invention includes, but is not limited to, competitive and non-competitive assay systems, using techniques such as BIAcore

analysis, fluorescence activated cell sorter (FACS) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art (see, for example, Ausubel et al., eds, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., NY (1994)).

An immunoassay of the present invention may be homogeneous or heterogeneous. A heterogeneous immunoassay requires a step to remove unbound antibody or antigen from the sample, usually using a solid phase reagent. Because homogeneous assays do not require this step, they are typically faster and easier to perform. Separation methods include, for example, precipitation (for example, with a second antibody) and removal on a coated tube, coated bead, coated well, magnetic particles or glass particles.

An immunoassay of the present invention may be, for example, a competitive binding assay. In a competitive immunoassay, the antigen in the sample competes with labeled antigen to bind with antibodies. The amount of labeled antigen bound to the antibody site is then measured. In this method, the response will be inversely proportional to the concentration of antigen in the unknown. This is because the greater the response, the less antigen in the sample was available to compete with the labeled antigen. An example of a competitive immunoassay is a radioimmunoassay (RIA).

An immunoassay of the present invention maybe be, for example, an Enzyme-Linked ImmunoSorbent Assay (ELISA). In an ELISA, an unknown amount of antigen is affixed to a surface and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal and the amount of antigen in the sample can be measured. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (for example, a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a

"sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. ELISAs may utilize chromogenic, luminescent, or fluorigenic substrates.

An immunoassay of the present invention may be a sandwich assay. In sandwich assays, the analyte is a "sandwich" between two antibodies that bind to different antigenic epitopes on the target analyte. One antibody serves as a capture antibody and a second antibody serves as a detection antibody. The capture antibody may be coated to a solid phase, such as a tube or well, and the detection antibody may be detectably labeled.

An immunoassay of the present invention may be, for example, an immunochromatographic lateral flow assay (also referred to herein as a lateral flow assay, a lateral flow test, or immunochromatographic strip test). Lateral flow assays use a simple device to quickly detect the presence (or absence) of a target analyte in sample. These tests are commonly used for medical diagnostics either for home testing, point of care testing, or laboratory use. Often produced in a dipstick format, lateral flow tests are a form of immunoassay in which the test sample, which may be suspended in an aqueous solution, flows through a porous substrate (for example, a nitrocellulose membrane) via capillary or wicking action towards an absorbent pad. After the sample is applied to the substrate, it encounters a colored reagent (for example, gold or latex particles) which mixes with the sample and binds to the analyate, if present in the sample. The mixture transits the substrate encountering lines or zones which have been pretreated to immobilize an antibody capable of binding the analyte. If the analyte is present in the sample, the colored reagent can become bound at the test line or zone. In alternative embodiments, the lateral flow assay can be used to detect specific antibodies present in a sample. In those embodiments, the colored reagent can be antigen-coated particles and the detection lines or zones can be pretreated with the antigen. See, for example U.S. Patent Nos. 5,753,517, 6,485,982,

6,509,196, 7,189,522, and RE39664, U.S. Patent Application 2006/0275920, and Jeong et al., 2003, *Korean J Biol Sci*; 7:89-92).

Also included in the present invention are detection methods in which one or more antibodies described herein are used to bind *C. difficile* spores, if present in a sample, to a substrate and the presence of spores is then detected and/or quantified by any of a variety of means. The presence of spores may be determined by, for example, microscopy, culturing, enzymatic activity antibody binding (as, for example, in an ELISA assay), calcium molecular fluorescence or luminescence, or lanthanide metal mediated luminescence. Dipicolinic acid in a 1:1 complex with calcium ions is present in high concentrations in bacterial spores and has not been observed in any other life forms. A lanthanide metal, such as for example, terbium or europium, will combine with dipicolinic acid (DPA) present in any bacterial spores in a sample to produce a lanthanate chelate, such as, for example, terbium or europium dipicolinate. Such lanthanate chelates have distinctive absorbance and emission spectra that can be detected using photoluminescence testing. Lanthanide metal mediated luminescence may also be utilized as a detection signal in any of the various immunoassay methods described herein, for example, in a lateral flow assay. Upon spore germination, Ca-DPA is released and calcium can be detected by a number of means. Fluorimetric detection of calcium by the use of molecular fluorescence or luminescence for sensing offers high sensitivity. Calcium indicator dyes can be categorized into two groups; the first are the dyes that increase their fluorescence in the presence of calcium, while the second group are dyes that have different excitation and/or emission wavelengths in the presence of calcium than they have in its absence. The calcium indicator dyes, calcium green-1, calcium green-2, and Fluo-4 are representative of the dyes that increase their fluorescence in the presence of calcium ion ($Ca2^{+}$) without changing wavelengths. Fura-2 and Indo-1 are ratiometric $Ca2^{+}$ indicators that are generally considered interchangeable in most experiments. Fura-2, upon binding $Ca2^{+}$, exhibits a shift in its absorption or excitation peak from 338 nm to 366 nm. Indo-1 on the other hand has a shift in the emission from 485 nm to 405 nm in the presence of calcium. Calcium can also be detected using calcium-activated photoproteins, such as aequorin and obelin. Since there is no need for excitation from external irradiation for the emission of bioluminescence, the signal produced has virtually no background. This allows for detection limits at extremely low levels, making these photoproteins attractive labels

for analytical applications. Calcium mediated signaling may also be utilized as a detection signal in any of the various immunoassay methods described herein, for example, in a lateral flow assay. Such methods, and the other methods described herein, also allow for the quantification of spores present in a sample. See, for example, U.S. Patent Nos. 5,876,960; 6,498,041; 6,815,178; and 7,306,942, U.S. Patent Application Serial Nos. 2003/0138876; 2004/0014154; and 2005/0136508; and Ponce, 2003, *NASA Tech Brief*; 27(3):pp. i-ii, 1-3.

With the detection methods of the present invention, one or more antibodies may be used, including one or more of the antibodies described herein that bind to a *C. difficile* spore. Further, one or more additional antibodies of known specificity may be used, for example, one or more antibodies that bind to *C. difficile* vegetative cells, that bind to vegetative cells of a different bacterial species, such as, for example, *C. clostridium* or *B. subtilis*, or that bind to spores of a different bacterial species, such as, for example, spores of *C. clostridium* or *B. subtilis*, may be used.

Samples may be obtained from a wide variety of source and include, but are not limited to, environmental or food samples and medical or veterinary samples. Examples of environmental samples, include, but are not limited to, water samples, soil samples, plant samples, and air samples. Examples of foods include, but are not limited to: meats, poultry, eggs, fish, seafood, vegetables, fruits, prepared foods (e.g., soups, sauces, pastes), grain products (e.g., flour, cereals, breads), canned foods, milk, other dairy products (e.g., cheese, yogurt, sour cream), fats, oils, desserts, condiments, spices, pastas, beverages, water, and animal feed. Medical or veterinary samples include, but are not limited to: clinical samples, cell lysates, whole blood or a portion thereof (e.g., serum), other bodily fluids or secretions (e.g., saliva, sputum, sweat, sebum, urine, cerebrospinal fluid), feces, cells, tissues, organs, biopsies, and different types of swabs.

A sample may be obtained from a fomite. The term "fomite" is generally used to refer to an inanimate object or substrate capable of carrying infectious organisms and/or transferring them. A fomite serves to transmit an infectious agent, such as *C. difficile*, from person to person. Fomites can include, but are not limited to, cloths, mop heads, towels, sponges, wipes, eating utensils, coins, paper money, cell phones, clothing (including shoes), doorknobs, feminine products, diapers, etc., portions thereof, and combinations thereof. There are many examples of fomites with respect to medicine; tools such as laryngoscopes that are not properly disinfected between uses,

dirty towels, eating utensils, and surfaces such as floors, walls, and tables may all serve to spread disease. The surface of interest can include at least a portion of a variety of surfaces, including, but not limited to, walls (including doors), floors, ceilings, drains, refrigeration systems, ducts (e.g., airducts), vents, toilet seats, handles, doorknobs, handrails, bedrails (e.g., in a hospital), countertops, tabletops, eating surfaces (e.g., trays, dishes, etc.), working surfaces, equipment surfaces, clothing, etc., and combinations thereof.

Samples may be liquid, solid, or semi-solid. Samples may be swabs of solid surfaces. Samples may be used directly in the detection methods of the present invention, without preparation or dilution. For example, liquid samples, may be assayed directly. Samples may be diluted or suspended in solution, which may include, but is not limited to a buffered solution or a bacterial culture medium. A sample that is a solid or semi-solid may be suspending in a liquid by mincing, mixing or macerating the solid in the liquid. A sample may by further concentrated or enriched.

The immunoassays of the present invention may include various appropriate control samples. For example, negative control samples containing no bacterial spores, cells or toxin, or positive control samples containing bacterial cells, spores or toxin may be assayed. An immunoassay of the present invention may take as little as a few minutes to develop and may require little or no sample or reagent preparation. An immunoassay of the present invention may be performed in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result for a sample. The cutoff between positive and negative is determined by the analyst and may be statistical. Two or three times the standard deviation is often used to distinguish positive and negative samples. In quantitative formats, results may be interpolated into a standard curve, which is typically a serial dilution of the target.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Generation of *C. difficile* spores

One tube of Brain Heart Infusion Broth (BHI) was inoculated with *C. difficile* (ATCC® No. 700792, American Type Culture Collection, Manassas, VA) and incubated for twenty-four hours at 35-37° C under anaerobic conditions. Following incubation, one milliliter (ml) aliquots of the broth culture were transferred to a minimum of four tubes containing BHI broth and then incubated for twelve days at 35-37° C under anaerobic conditions. The broth cultures were centrifuged at 10,000 rotations per minute (rpm) for 10 minutes (min) and the cell pellet was resuspended in 10 ml of absolute ethanol for one hour at room temperature to kill vegetative cells. After one hour the suspension was centrifuged at 10,000 RPM for 10 minutes and the cell pellet was washed at least twice with sterile Butterfield's buffer. The pellet was resuspended in sterile Butterfield's Buffer and spore number per milliliter was determined by plating serial dilution on anaerobic blood agar. Resuspended spores were used in the following examples as both an immunogen in the generation of polyclonal antibodies to inactivated spores and as an antigenic target in ELISA assays to determine the binding specificity of anti-spore antibodies.

Example 2

Generation of polyclonal antibody to inactivated *C. difficile* spores

C. difficile spores, at a concentration of about 10^6 spores per ml, were treated with 5% formalin for 10 minutes to inactivate the spores. The inactivated spores were washed twice with sterile Butterfield's buffer and then resuspended in sterile Butterfield's buffer. A polyclonal antibody was raised against the inactivated spores in rabbits using standard protocols (Antagene, Inc, Mountain View, CA). Briefly, New Zealand White rabbits (two individual rabbits) were immunized with 1 x 10^6/ml equivalent of inactivated spores per immunization. The immunogen was diluted to 1 ml with sterile saline and combined with 1 ml of the appropriate adjuvant. The antigen and adjuvant were mixed to form a stable emulsion which was injected subcutaneously. Rabbits were immunized on day one with antigen in Complete Freund's Adjuvant (CFA), followed by immunizations on days 20, 40, and 60 with antigen in Incomplete Freund's Adjuvant (IFA) for all subsequent injections.

Ten days after the last immunization, the blood was collected from the rabbit and allowed to clot and retract at 37°C overnight. The clotted blood was then refrigerated for 24 hours and the serum was decanted and clarified by centrifugation at 2500 rpm for 20 minutes. The preimmune and immune serums were tested against inactivated spores by ELISA. For the ELISA protocol, inactivated spores of *C. difficile* were diluted to 10^5 spores per milliliter (ml) in coating buffer (0.1 M bicarbonate buffer 1.59 grams (g) Na_2CO_3 and 2.93g $NaHCO_3$ per liter of sterile distilled water, pH 9.6). 100 microliter (µl) of the spore solution was added to wells of an ELISA plate (ELISA Enhanced Surface plate, BD Falcon, Franklin Lakes, NJ). To the control wells, 100 µl of coating buffer was added. The plates were wrapped with PARAFILM and incubated at 4° C for 15 to 16 hours. The plates were emptied and washed three times with wash buffer (Phosphate buffered saline with 0.05% Tween 20). The plates were blocked with 100 µl of blocking buffer (1% BSA and 0.05% Tween-20 in PBS) for two hours with shaking at room temperature (RT). The plates were emptied, washed three times with wash buffer and incubated with 100 µl primary antibody in blocking buffer for one to two hours with shaking at RT. The plates were emptied, washed three times with wash buffer and incubated with 100 µl of secondary antibody with HRP label (goat anti-rabbit HRP conjugate; 1:10,000 dilution, Pierce, Rockford, IL) for one hour with shaking at RT. The plates were emptied and washed four times with wash buffer. 50 µl of the substrate solution (1-step Ultra TMB, Pierce, Rockford, IL) was added to each of the wells and incubated with shaking at RT for 15 to 30 minutes. The color was stopped by adding 50 µl of stop solution (1.5 M phosphoric acid) and absorbance of each plate was read in a SpectraMax plus 384 (Molecular Devices, Sunnyvale, CA) at 450 nm. The absorbances were compared to determine fold-enhancement of signal for target. The immunized serum showed a good response to inactivated spores, approximately 13 to 15 fold that obtained with the preimmune serum (Table 1).

Table 1. Characterization of antiserum generated against inactivated *C. difficile* spores

Absorbance at 450 nm*				
Antibody dilution	Rabbit #1		Rabbit #2	
	Preimmune serum	Antisera	Preimmune serum	Antisera
1:1000	0.07	1.73	0.04	1.92
1:10,000	0.06	1.22	0.07	1.27

1:100,000	0.06	0.78	0.06	0.82

*Average no antigen control readings with preimmune and antiserum were 0.12 at 450 nm.

The antibody was purified by ammonium sulfate precipitation followed by protein A column chromatography. Briefly, antisera were precipitated by drop-wise addition of ice-cold saturated ammonium sulfate to a final ratio of 1:1 (50% saturated ammonium sulfate). This procedure was done in an ice-cold beaker, with constant stirring. The supernatant was transferred to a 50-mL centrifuge tube and placed on a reciprocating mixer overnight at 4°C. The suspension was centrifuged at 10,000 x g for 30 minutes at 2°C. The supernatant was removed and the pellet was resuspended in an equal volume of deionized water. The resuspended protein was dialyzed (12,000-14,000 MW cut-off dialysis tubing) against two liters (L) of PBS. The dialysis buffer was removed and replaced after about two hours and again after about 24 hours. After about 48 hours of dialysis, the protein dialysate was removed and filtered with a 0.22 μm filter.

The IgG fraction was recovered from the filtrate by chromatography, using a Protein A affinity column from BioRad (Hercules, CA) according to the manufacturer's instructions. Briefly, a Shimadzu HPLC system (Model SCL-10AVP, Shimadzu Corporation, Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for all preparative chromatography runs. The solvents used for the binding and elution of the antibodies are listed below were Binding Buffer A (Phosphate Buffered Saline, pH 7.3) and Elution Buffer B (20 mM Sodium Acetate, 0.5 M NaCl, pH 3.5). All buffers were prepared with deionized water using a MILLI Q filtration system (Millipore Corp., Billerica, MA) and were filtered through a 0.22-μm (pore size) membrane filter. The column was pre-equilibrated with binding buffer A prior to sample injection. The antisera samples were manually injected into the column via a sample injection loop at T=0 minutes. After sample injection, the solvents were run through the column as follows: Binding Buffer B starting at T=0 at a flow rate of 0.6ml/min; Elution Buffer B starting at T=20 minutes at a flow rate of 1.0ml/min; and Binding Buffer B starting at T=60 minutes at a flow rate of 1.0ml/min. Fractions of the column eluate from each of the mobile phase solvents were collected. Purified anti-*C. difficile* antibody protein eluted at around 51 minutes. Each run was 70 minutes long after which the second peak was put into dialysis for three changes of buffer to change the pH. The fractions

were then filtered and pooled and the OD measured. At 20 minutes the buffer was changed to elution buffer. The purified antibody was dialysed against PBS with three changes of buffer at 4°C. The dialysed antibody was concentrated using Centricon filters (10,000 molecular weight cut off; Millipore Corporation, Billerica, MA)

The purified antibody was tested by ELISA against *C. difficile*, *C. sporogenes* (ATCC 3584) and *B. subtilis* (ATCC 19659) spores. The *C. sporogenes* and *B. subtilis* spores were obtained from Presque Isle Cultures (Erie, PA). Spores were diluted in coating buffer. Various levels of spores (10^3 to 10^5 per ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated and tested with the antibody in ELISA assays performed as described above. The antigen antibody interaction was detected using anti-rabbit HRP antibody (Pierce, Rockford, IL) and the spore antibody showed good reaction with *C. difficile* spores, about 20-fold response over background at 10^5 spores per ml, and showed a weak, approximately 2-fold with *C. sporogenes*, and no reaction over background with *B. subtilis* spores (see Table 2). The data presented in Table 2 are the average of readings from three wells from one of the experiment and are representative of at least three separate experiments.

Table 2. Characterization of purified rabbit *C. difficile* spore antibody

	Absorbance at 450 nm		
Spores/ml	*B. subtilis*	*C. sporogenes*	*C. difficile*
1.00E+03	0.21	0.24	0.17
1.00E+04	0.16	0.25	0.8
1.00E+05	0.16	0.41	4.13
No antigen control	0.17	0.17	0.17

Example 3

Selection of *C. difficile* spore-specific proteins for generation of antibodies

The complete genome sequence of *C. difficile* strain 630 has been determined and is available on the GenBank® sequence database maintained by the National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), National Institutes of Health (NIH). See also, Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786). A thorough search of all the GenBank® entries for *C. difficile* was made and two spore

specific proteins were picked for antibody production. One of the proteins selected was hypothetical protein CD1021 (YP_001087502), which demonstrates a conserved domain (amino acid residues 90 to 393) to the spore coat assembly protein H (cotH). The other protein selected was cell surface protein (putative N-acetylmuramoyl-L-alanine amidase, YP_001087517) which has conserved domains CW_binding_2 (putative cell wall binding repeat 2; 174 to 265, 275 to 368, 381 to 461) and Amidase_3 (N-acetylmuramoyl-L-alanine amidase, 493 to 673). The analysis was performed using conserved domain search tools available from the NCBI and available on the worldwide web at ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml.

Hypothetical Protein CD1021 of *C. difficile 630* (GenBank Accession No. YP_001087502) has the following amino acid sequence:

MKDKKFTLLI SIMIVFLCAV VGVYSTSSNK SVDLYSDVYI EKYFNRDKVM
EVNIEIDESD LKDMNENAIK EEFKVAKVTV DGDTYGNVGI RTKGNSSLIS
VANSDSDRYS YKINFDKYNT SQSMEGLTQL NLNNCYSDPS YMREFLTYSI
CEEMGLATPE FAYAKVSING EYHGLYLAVE GLKESYLENN FGNVTGDLYK
SDEGSSLQYK GDDPESYSNL IVESDKKTAD WSKITKLLKS LDTGEDIEKY
LDVDSVLKNI AINTALLNLD SYQGSFAHNY YLYEQDGVFS MLPWDFNMSF
GGFSGFGGGS QSIAIDEPTT GNLEDRPLIS SLLKNETYKT KYHKYLEEIV
TKYLDSDYLE NMTTKLHDMI ASYVKEDPTA FYTYEEFEKN ITSSIEDSSD
NKGFGNKGFD NNNSNNSDSN NNSNSENKRS GNQSDEKEVN AELTSSVVKA
NTDNETKNKT TNDSESKNNT DKDKSGNDNN QKLEGPMGKG GKSIPGVLEV
AEDMSKTIKS QLSGETSSTK QNSGDESSSG IKGSEKFDED MSGMPEPPEG
MDGKMPPGMG NMDKGDMNGK NGNMNMDRNQ DNPREAGGFG NRGGGSVSKT
TTYFKLILGG ASMIIMSIML VGVSRVKRRR FIKSK (SEQ ID NO:1). See also, Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786 and the worldwide web at ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id=126698605.

The putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* 630 (GenBank Accession No. YP_001087517) has the following amino acid sequence:

MLSKEINMRR NTKLLTTGIL SMAIVAPTMA FATESNAMEN NADLNINLEK
KSIVLGSKSK VSVKFKEKPD ADSIKLKYKC YDMPLNTTLN YNQSTGAYEG
IINYNKDPEY LNVWELQGIT INSKTNPKTL NRQDLEKMGL NLKDYNVTQE
CIIEDITSRK DVNKYLRKTS SPITELTGSD RYETAVKISK EGWKNGSDKV

VIINGDVSID GIISTPLATT YNAPILLVEK NNVPNSVKSE LKRLNPKDII
IIGDENAISK TTANQIKSTV NASQTRLNGS NRYETSLLIA KEIDKNHDVE
KVYITNANGG EVDALTIAAK AGQDKQPIIL TDKDSITDNT YKWLKSEDLQ
NAYFIGGPQM ISTNVINKVN GITKDSVTNN RVYGADRHET NANVIKKFYT
DDELEAVLVA KSDVLVDALA AGPLAANLKS PILITPKTYV SAYHKDNLEA
KSANKVYKIG GGLTSKVMSS IASSLSKHNT TPTEPGNSGG KTVMIDPGHG
GSAPGNSSGG MIEKDYNLNT SLATTEYLRS KGFNVIMTRD TDKTLSLGNR
TALSNSLKPD LFTSIHYNGS TNKQGHGVEV FYKLKDKNGG TTKTVATNIL
NRILEKFKLT NRGIKTRVLP SDSTKDYLYV LRSNDMPAVL VECAFLDNEN
DMSLINSSAK VKEMGTQIGK GIEDSLK (SEQ ID NO:5). See also, Sebaihia et al., 2006, *Nat. Genet*; 38 (7):779-786 and the worldwide web at ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&id.

Example 4

GAT polyclonal antibody against hypothetical protein CD1021

A polyclonal antibody against hypothetical protein CD1021 was generated using Strategic Diagnostics Inc.'s (SDI) (Newark, DE) proprietary Genomic Antibody Technology™ (GAT). A unique amino acid sequence from the protein was identified and this sequence information was placed in SDI's proprietary plasmid vector. The vector was introduced into a mouse. With this technology, cells of the host animal take up the plasmid. In these cells, the immunogen is synthesized and secreted by the host cells and immediately recognized by the immune system leading to production of polyclonal antibodies against the expressed protein sequence. Both polyclonal and monoclonal antibodies can be developed using GAT. The protein immunogen is produced in the host animal using natural protein synthesis machinery. Since the immunogen is not synthesized and purified in a laboratory, it does not have the opportunity to denature or degrade. A native immunogen is presented immediately to the immune system, resulting in a mature antibody response.

The protein sequence of hypothetical protein CD1021 used for polyclonal antibody generation was residues 505 to 604 and had the amino acid sequence:
SKTIKSQLSG ETSSTKQNSG DESSSGIKGS EKFDEDMSGM PEPPEGMDGK
MPPGMGNMDK GDMNGKNGNM NMDRNQDNPR EAGGFGNRGG GSVSKTTTYF

(SEQ ID NO:2).

The sera from two immunized mice was pooled and tested against the immunogen by Western blot to determine specificity of the antibody. Later, the antibody was purified by ammonium sulfate precipitation followed by protein A column chromatography, as described in Example 2.

Initially, various levels of spores (10^3 to 10^5 per ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated and tested with the antibody. The antigen-antibody interaction was detected using anti-mouse HRP antibody (Pierce, Rockford, IL). The mouse antibody against the hypothetical protein CD1021 (SEQ ID NO:2) showed about a 10-fold increased response over background at 10^5 spores per ml, and showed a weak response (about 2-fold) with *C. sporogenes* spores, and no reaction over background with *B. subtilis* spores (see Table 3). The data presented in Table 3 are the average of readings of three wells and are representative of two separate experiments.

Table 3. Characterization of *C. difficile* hypothetical protein CD1021 mouse antibody

Absorbance at 450 nm			
Spores/ml	*B. subtilis*	*C. sporogenes*	*C. difficile*
1.00E+03	0.13	0.13	0.2
1.00E+04	0.15	0.17	0.2
1.00E+05	0.15	0.32	1.61
No antigen control	0.14	0.14	0.14

The antibody was further tested using sandwich ELISA. Plates were coated with the polyclonal CD1021 antibody followed by binding of spores (*B. subtilis*, *C. sporogenes* and *C. difficile*). The antibody antigen interaction was detected using the anti-*C. difficile* inactivated spore antibody from example 2, above, as the second antibody, followed by anti-rabbit HRP antibody (BD Pharmingen). The spores of *B. subtilis*, *C. sporogenes*, and *C. difficile* were used at various levels and antibody is specific for detection of *C. difficile* spores. The results are shown in Table 4, below.

In the sandwich ELISA protocol, the purified mouse CD1021 antibody was diluted in antigen coating buffer at 1 μg/ml concentration. 100 μl of the of the antibody solution was added to wells of an ELISA plate (ELISA Enhanced Surface plate, BD Falcon, Franklin Lakes, NJ). The plates were wrapped with Parafilm and incubated at

4° C for 15 to 16 hours. The plates were emptied and washed four times with wash buffer. The plates were blocked with 100 µl of blocking buffer for 2 hours with shaking at room temperature (RT). The plates were emptied, washed four times with wash buffer and spore solutions of *C. difficile*, *C. sporogenes*, *and B. subtilis* (100 µl of 10^3, 10^4, and 10^5 spores per ml) in blocking buffer were added. For control wells, 100 µl of blocking buffer was added. After incubation at RT for two hours, the plates were emptied, washed four times with wash buffer and incubated with 100 µl of secondary antibody (anti-rabbit *C. difficile* spore antibody, descried in Example 2) in blocking buffer for one hour with shaking at RT. The plates were emptied, washed four times with wash buffer and incubated with 100 µl of secondary antibody with HRP label (goat anti-rabbit HRP conjugate; 1;10,000 dilution, BD Pharmingen) for one hour with shaking at RT. The plates were emptied and washed three times with wash buffer. 50 µl of the substrate solution (1-step Ultra TMB, Pierce) was added to each of the wells and incubated with shaking at RT for 15 to 30 minutes. The color was stopped by adding 50 µl of stop solution (1.5 M phosphoric acid) and absorbance of each plate was read at 450 nm. The absorbances were compared against control to determine fold-enhancement of signal for target (see Table 4). The data presented in Table 4 are the average of readings of three wells and are representative of two separate experiments.

Table 4. Characterization of *C. difficile* hypothetical protein CD1021 mouse antibody by Sandwich ELISA with spore antibody

Fold-change over control*			
Spores/ml	*B. subtilis*	*C. sporogenes*	*C. difficile*
1.00E+03	1.1	1.03	1.22
1.00E+04	1.12	1.08	1.4
1.00E+05	1.14	1.04	2.35

*No antigen control readings were 1.05 at 450 nm

Example 5

Polyclonal antibodies against hypothetical protein CD1021 peptides

Two unique amino acid sequences were identified from the hypothetical protein CD1021. A search in both protein and DNA databases showed that both sequences are

specific to *C. difficile* and have no homology with other bacteria such as *Bacillus.* These two sequences are:

Peptide 1 EGSSLQYKGDDPESY (SEQ ID NO:3)
(residues 203 to 217 of CD1021)

Peptide 2 LKNETYKTKYHKYLE (SEQ ID NO:4)
(residues 333 to 347 of CD1021).

The peptides were synthesized with a free cysteine at the N-terminal end and conjugated to keyhole limpet hemocyanin (KLH) to elicit high titer antibodies. Each of the two KLH conjugated peptides was used separately to immunize two individual rabbits according to standard protocols with antigen by Epitomics (Burlingame, CA). Briefly, a primary injection of KLH-conjugated peptide (0.5mg/ml) with 1ml of CFA was followed by four boosts of KLH-conjugated peptide (0.25mg/ml) with 1ml of IFA.

Blood was collected after each immunization and sera was tested by ELISA using the respective peptides. Bleed three and four showed a good response to the peptides, approximately 5 to 7-fold response over the preimmune serum (Table 5).

Table 5. Characterization of *C. difficile* CD1021 peptide serum (bleed 3)

Absorbance at 450 nm*									
	Peptide 1				Peptide 2				
Antibody dilution	Rabbit #1		Rabbit #2		Rabbit #1		Rabbit #2		
	Preimmune serum	Antisera	Preimmune serum	Antisera	Preimmune serum	Antisera	Preimmune serum	Antisera	
1:1000	0.18	1.72	0.20	1.62	0.200	1.82	0.20	1.81	
1:10,000	0.15	1.21	0.15	1.12	0.120	1.18	0.14	1.05	
1:100,000	0.10	0.68	0.08	0.72	0.070	0.64	0.08	0.74	

* Average no antigen control readings with preimmune and antiserum were 0.15 at 450 nm.

The serum from bleed three and four were purified by ammonium sulfate precipitation followed by protein A column chromatography, as described in Example 2. The purified antibody was tested by ELISA for binding to plate bound peptides.

The peptides were diluted in antigen coating buffer at 1 μg/ml concentration. 100 μl of the of the peptide solution was added to wells of an ELISA plate (ELISA Enhanced Surface plate, BD Falcon, Franklin Lakes, NJ). To the control wells 100 μl of coating buffer was added. The plates were wrapped with Parafilm and incubated at 4° C for 15 to 16 hours. The plates were emptied and washed three times with wash buffer. The plates were blocked 100 μl of blocking buffer for two hours with shaking at room temperature (RT). The plates were emptied, washed three times with wash buffer and incubated with 100 μl primary antibody in blocking buffer for one to two hours with shaking at RT. The plates were emptied, washed three times with wash buffer and incubated with 100 μl of secondary antibody with HRP label (1;10,000 dilution) for 1 hour with shaking at RT. The plates were emptied and washed three times with wash buffer. 50 μl of the substrate solution (1-step Ultra TMB, Pierce) was added to each of the wells and incubated with shaking at RT for 15 to 30 minutes. The color was stopped by adding 50 μl of stop solution (1.5 M phosphoric acid) and absorbance of each plate was read at 450 nm. The absorbances were compared against control to determine fold-enhancement of signal for target.

Various levels of spores (10^3 to 10^5 per ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated and tested with the antibody. The antigen antibody interaction was detected using anti-rabbit HRP antibody (Pierce, Rockford, IL) and the antibodies were specific for detection of *C. difficile* spores, demonstrating a 6 to 10-fold response over background with *C. difficile* spores (see Table 6). The data presented in Table 6 are the average of readings of three wells and are representative of at least three separate experiments.

Table 6. Characterization of rabbit antibodies to peptide sequences of *C. difficile* hypothetical protein CD1021

Absorbance at 450 nm						
	Peptide 1 Ab			Peptide 2 Ab		
Spores/ml	*B. subtilis*	*C. sporogenes*	*C. difficile*	*B. subtilis*	*C. sporogenes*	*C. difficile*
1.00E+03	0.15	0.14	0.2	0.14	0.14	0.21
1.00E+04	0.16	0.17	0.21	0.17	0.17	0.48
1.00E+05	0.16	0.29	1.53	0.16	0.27	0.95
No Antigen control	0.22	0.22	0.22	0.2	0.2	0.2

Example 6

GAT polyclonal antibody against a putative N-acetylmuramoyl-L-alanine amidase cell surface protein

A polyclonal antibody against a cell surface protein that is a putative N-acetylmuramoyl-L-alanine amidase protein was generated using Strategic Diagnostics Inc.'s (Newark, DE) proprietary Genomic Antibody Technology™ (GAT). A unique sequence from the protein was identified and the immunogen was expressed in vivo in mouse, as described in Example 4. The expressed immunogen is recognized by the host immune system leading to production of a polyclonal antibody against the expressed protein. The protein sequence of cell surface protein (putative N-acetylmuramoyl-L-alanine amidase) used for antibody generation was residues 294 to 393, having the amino acid sequence of DKNHDVEKV YITNANGGEV DALTIAAKAG QDKQPIILTD KDSITDNYKW LKSEDLQNAY FIGGPQMIST NVINKVNGIT KDSVTNNRVY GADRHETNAN (SEQ ID NO:6).

The sera was pooled from immunized animals and tested against the immunogen by Western blot to determine specificity of the antibody. Later, the antibody was purified by ammonium sulfate precipitation followed by protein A column chromatography.

Initially, various levels of ungerminated spores (10^3 to 10^5 per ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated and tested with the antibody. The antigen antibody interaction was detected using anti-mouse HRP antibody (Pierce, Rockford, IL). The antibody against cell surface protein (putative N-acetylmuramoyl-L-alanine amidase) did not show any reaction with C. difficile or other spores.

The spores of *B. subtilis*, *C. sporogenes* and *C. difficile* were germinated using various germinant solutions. The germinated spores (10^3 to 10^5 per ml) were coated and tested with the antibody. The antigen antibody interaction was detected using anti-mouse HRP antibody (Pierce, Rockford, IL). The antibody was able to detect germinated *C. difficile* spores, demonstrating about a 6-fold response over background, but not germinated *B. subtilis* or *C. sporogenes* spores (see Table 7). The data presented in Table 7 are the average of readings of three wells and are representative of two separate experiments.

Germination of spores has been well studied with *B. subtilis* spores which can be induced to germinate by specific germinants, including L-alanine and a combination of asparagine glucose, fructose and potassium ions ("AGFK") (Moir and Smith, 1990, *Ann Rev Microbiol*; 44:531-553). Initial attempts to germinate *C. difficile* spores were using various germinants (combination of AGFK with alanine and Inosine), but spore germination was not efficient. The *C. difficile* spores germinate in a nutrient medium with addition of 1% sodium taurocholate (Sorg and Sonenshein, 2008, *J Bacteriol* (published online ahead of print on 1 February 2008) doi:10.1128/JB.01765-07), the spores were germinated in Brain Brain-Heart Infusion broth supplemented with yeast extract (5 mg/ml), L-cysteine (0.1%) and 1% sodium taurocholate. The germination of spores was followed by measuring the OD600 of spore cultures (OD600 decreases upon germination) and by phase contrast microscopy. Spores of *C. sporogenes* and *B. subtilis* were germinated following previously as described before (Broussolle et. al., 2002, *Anaerobe*; 8:89-100; Moir and Smith, 1990, *Ann Rev Microbiol*; 44, 531-553).

Table 7. Characterization of *C. difficile* putative amidase mouse antibody

Absorbance at 450 nm						
Spores/ml	*B. subtilis*		*C. sporogenes*		*C. difficile*	
	Ungerminated	Germinated	Ungerminated	Germinated	Ungerminated	Germinated
1.00E+03	0.13	0.17	0.14	0.16	0.2	0.18
1.00E+04	0.12	0.2	0.13	0.22	0.2	0.39
1.00E+05	0.15	0.22	0.15	0.27	0.2	0.82
No antigen control	0.13	0.13	0.13	0.13	0.13	0.13

Example 7

Polyclonal antibody against cell surface protein
(putative N-acetylmuramoyl-L-alanine amidase) peptides

Two unique sequences were identified from the protein sequence of *C. difficile* N-acetylmuramoyl-L-alanine amidase and upon BLAST search in both protein and DNA database the sequences are specific to *C. difficile* and have no homology with other bacteria such as *Bacillus*.

Peptide 1 YKLKDKNGGTTKTVA (SEQ ID NO:7) (amino acid residues 582 to 596)

Peptide 2 KFKEKPDADSIKLKY (SEQ ID NO:8) (amino acid residues 64 to 78)

The peptides were synthesized with a free cysteine at the N-terminal end and conjugated to KLH to elicit high titer antibodies. Both KLH conjugated peptides were combined and used together to immunize two individual rabbits according to standard protocols by Antagene (Mountain View, CA). Ten days after the last immunization, the blood was collected from the rabbit and allowed to clot and retract at 37°C overnight. The clotted blood was then refrigerated for 24 hours and the serum was decanted and clarified by centrifugation at 2500 rpm for 20 minutes. Initial ELISA was done with preimmune and immunized serum against the peptides. Immunized serum against the peptides showed good response to peptides, demonstrating about a 7 to 10-fold response over the preimmune serum (Table 8).

Table 8. Characterization of *C. difficile* putative amidase peptide serum

Absorbance at 450 nm*				
Antibody dilution	Rabbit #1		Rabbit #2	
	Preimmune serum	Antisera	Preimmune serum	Antisera
1:1000	0.25	1.83	0.22	1.70
1:10,000	0.20	1.55	0.15	1.81
1:100,000	0.12	0.91	0.10	0.98

*Average no antigen control readings with preimmune and antiserum were 0.20 at 450 nm.

The antibody was purified by ammonium sulfate precipitation followed by protein A column chromatography, as described in Example 2. The purified antibody was tested by ELISA. Initially, various levels of ungerminated spores (10^3 to 10^5 per

ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated and tested with the antibody. The antigen antibody interaction was detected using anti-rabbit HRP antibody (Pierce, Rockford, IL). The antibody against cell surface protein (putative N-acetylmuramoyl-L-alanine amidase) did not show any reaction with *C. difficile* or other spores. The spores of *B. subtilis*, *C. sporogenes* and *C. difficile* were germinated using various germinant solutions. The germinated spores (10^3 to 10^5 per ml) were coated and tested with the antibody. The antigen antibody interaction was detected using anti-rabbit HRP antibody (Pierce, Rockford, IL). The antibody was able to detect germinated *C. difficile* spores, demonstrating about a 6-fold response over background, but not *B. subtilis* or *C. sporogenes* germinated spores (see Table 9). The data presented in Table 9 are the average of readings of three wells and are representative of two separate experiments.

Table 9. Characterization of *C. difficile* putative amidase rabbit antibody

Absorbance at 450 nm						
Spores/ml	*B. subtilis*		*C. sporogenes*		*C. difficile*	
	Ungerminated	Germinated	Ungerminated	Germinated	Ungerminated	Germinated
1.00E+03	0.13	0.18	0.14	0.17	0.2	0.18
1.00E+04	0.13	0.19	0.12	0.21	0.18	0.44
1.00E+05	0.14	0.22	0.15	0.26	0.19	0.93
No antigen control	0.13	0.13	0.13	0.13	0.13	0.13

Example 8

Polyclonal antibody against *C. difficile* common antigen for detection of *C. difficile* spores

The commercially available rabbit *C. difficile* common antigen (glutamate dehydrogenase) antibody (Meridian Life Science, Saco, ME) reacts with both toxigenic and nontoxigenic strains and is used to detect vegetative cells of *C. difficile*. In this example, the commercially available rabbit *C. difficile* common antigen antibody was tested for detection of *C. difficile* spores by ELISA. The antibody was purified by ammonium sulfate precipitation followed by protein A column chromatography. Various levels of spores (10^3 to 10^5 per ml) of *B. subtilis*, *C. sporogenes* and *C. difficile* were coated to a plate and tested with the antibody. The antigen antibody interaction was detected using anti-rabbit HRP antibody (Pierce, Rockford, IL). The common antigen antibody showed good reaction with *C. difficile* spores (about a 10-fold response with 10^4 spores per ml and an 18-fold response over background at 10^5 spores per ml) and showed a weak (about 2-fold response over background) with *C. sporogenes* spores and no reaction over background with *B. subtilis* spores (see Table 10). The data presented in Table 10 are the average of readings of three wells and are representative of at least three separate experiments. This antibody may be used as a capture antibody in sandwich ELIZA assays.

Table 10. Characterization of rabbit *C. difficile* common antigen antibody

Absorbance at 450 nm			
Spores/ml	*B. subtilis*	*C. sporogenes*	*C. difficile*
1.00E+03	0.14	0.15	0.44
1.00E+04	0.15	0.15	2.13
1.00E+05	0.2	0.31	3.69
No antigen control	0.19	0.19	0.19

Example 9

Binding of *C. difficile* antibodies to vegetative cells

The rabbit polyclonal antibody to inactivated spores (described in more detail in Example 2), rabbit polyclonal antibody to CD1021 sequence SEQ ID NO:3 (described in more detail in Example 5), rabbit polyclonal antibody to the two amidase peptides SEQ ID NO:7 and SEQ ID NO:8 (described in more detail in Example 7), and the commercially available anti-GDH antibody (described in more detail in Example 8) were screened by ELISA for binding to vegetative cells of *C. difficile*. *C. difficile* ATCC strains 43594, 43596, and 43603 were grown in thioglycollate medium under anaerobic conditions for 16 to 18 hours. The cells were serially diluted in antigen coating buffer and 100 µl of various dilutions was added to the ELISA plate and the plate was incubated under anaerobic condition for one hour at 37° C, to prevent sporulation. The plates were emptied and washed three times with wash buffer and blocked with blocking buffer for one hour under anaerobic conditions at 37° C. The plate was emptied and washed three times with wash buffer and incubated with primary antibody at RT for one hour. The plates were emptied, washed three times with wash buffer, incubated with secondary antibody with HRP label (1:10,000) for one hour at RT. The plates were washed and substrate was added to develop color and plates were read at 450 nm after adding the stop solution. The rabbit polyclonal antibodies to inactivated spores, CD1021 (SEQ ID NO:3) and amidase (SEQ ID NO:7 and SEQ ID NO:8) did not show any binding with vegetative cells (about 10^3 to 10^7 cells per ml). However, the commercially available anti-GDH antibody showed good binding at about 10^7 cells per ml, with a four-fold enhancement of signal over control (see Table 11). The data presented in Table 11 are the average of readings of three wells and are representative of two separate experiments.

Table 11. Binding of antibodies with vegetative cells.

ATCC 43594						
Absorbance at 450 nm						
Antibody	No antigen control	10^3 cells/ml	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml	10^7 cells/ml
Spore Ab	0.12	0.11	0.13	0.12	0.12	0.16
Amidase Ab	0.15	0.14	0.11	0.11	0.12	0.27
CD1021 peptide 1 Ab	0.11	0.11	0.11	0.12	0.12	0.24
GDH Ab	0.12	0.11	0.10	0.14	0.35	0.85

ATCC 43596						
Absorbance at 450 nm						
Antibody	No antigen control	10^3 cells/ml	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml	10^7 cells/ml
Spore Ab	0.12	0.12	0.12	0.11	0.11	0.14
Amidase Ab	0.15	0.11	0.11	0.11	0.12	0.22
CD1021 peptide 1 Ab	0.11	0.11	0.12	0.11	0.13	0.21
GDH Ab	0.12	0.11	0.12	0.16	0.40	0.76

ATCC 43603						
Absorbance at 450 nm						
Antibody	No antigen control	10^3 cells/ml	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml	10^7 cells/ml
Spore Ab	0.12	0.12	0.13	0.12	0.12	0.15
Amidase Ab	0.15	0.11	0.12	0.11	0.13	0.25
CD1021 peptide 1 Ab	0.11	0.11	0.12	0.11	0.12	0.22
GDH Ab	0.12	0.09	0.10	0.14	0.43	0.80

Example 10

Use of antibodies in lateral flow devices for *C. difficile* spore detection

Various antibodies described in the above examples, were labeled with Cy3 (Cy3 Ab labeling kit, Amersham Biosciences, Piscataway, NJ) and tested for ability to detect *C. difficile* spores. Specifically, the rabbit polyclonal antibody to inactivated spores (described in more detail in Example 2 and referred to as "spore" antibody in Table 12), the rabbit polyclonal antibody to CD1021 sequence SEQ ID NO:3 (described in more detail in Example 5 and referred to as CD1021 peptide 1 Ab in Table 12), the rabbit polyclonal antibody to a mix of the two amidase peptides SEQ ID NO:7 and SEQ ID NO:8 (described in more detail in Example 7 and referred to as amidase peptide Ab in Table 12), and the commercially available rabbit antibody to *C. difficile* glutamate dehydrogenase common antigen (described in more detail in Example 8 and referred to as "GDH" antibody in Table 12) were labeled and tested.

A typical lateral flow strip with conjugate pad, nitrocellulose membrane, and absorbent pad was prepared. Antibodies were spotted on the nitrocellulose and allowed to dry. Germinated and ungerminated spores (10^5 per ml) of *C. difficile*, *C. sporogenes* and *B. subtilis* were mixed with of each of the labeled antibodies (50 µl of 10 µg/ml

Cy3 labeled antibody) separately and applied to the conjugate pad. The antibody-spore mixture was allowed to wick for ten minutes. Phosphate buffered saline was used as a control. After ten minutes, the strips were scanned using a microarray scanner (Tecan, Durham, NC).

The lateral flow strips using the *C. difficile* spore antibody, amidase antibody, or the CD1021 antibody, showed that the antibodies are specific in detecting *C. difficile* spores. The amidase antibody did not detect ungerminated spores, but was able to detect germinated spores. Based on these observations one can design lateral flow strips for detecting *C. difficile* spores. For example, labeled GDH antibody could be used as a detection reagent and spore antibody or CD1021 antibody as capture reagent to detect ungerminated spores and amidase antibody as capture reagent to detect germinated spores.

As outlined in Table 12, various pairings of antibodies can be used to detect the *C. difficile* spores, detecting, spores or germinated spores.

Antibody 1	Antibody2	Specificity
Amidase Peptide Ab	GDH	*C. difficile* germinated spores
Amidase Peptide Ab	CD1021 peptide 1 Ab	*C. difficile* germinated spores
Amidase Peptide Ab	Spore	*C. difficile* germinated spores
CD1021 peptide 1 Ab	GDH	*C. difficile* spores
CD1021 Peptide 1 Ab	Amidase Peptide Ab	*C. difficile* germinated spores
CD1021 Peptide 1 Ab	Spore	*C. difficile* spores
Spore	GDH	*C. difficile* spores
Spore	Amidase Peptide Ab	*C. difficile* germinated spores
Spore	CD1021 Peptide 1 Ab	*C. difficile* spores
Spore	Spore	*C. difficile* spores
CD1021 Peptide 1 Ab	CD1021 Peptide 1 Ab	*C. difficile* spores
Amidase Peptide Ab	Amidase Peptide Ab	*C. difficile* germinated spores

Table 12. Antibody pairings for detection of *C. difficile* spores

Example 11

Detection of *C. difficile* spores on a surface

100 µl 10^6 per ml of *C. difficile* spores were spread on sterile aluminum coupons (1" x 3") and allowed to dry at RT for about an hour. The spores were recovered by rubbing vigorously for 5 to 10 seconds with a sterile swab moistened with about 50 µl of sterile PBS. The swabs containing the spores were immersed in 1 ml of coating buffer and vortexed vigorously for one to two minutes. The cotton swab was removed from the solution and recovery of spores was determined by ELISA. For control, the coupons were spread with sterile PBS and processed similarly. As seen in Table 13, the *C. difficile* spore antibody and the CD1021 peptide 1 Ab were able to detect the presence of spores on a surface.

Table 13. Detection of *C. difficile* spores from aluminum coupons

Absorbance at 450 nm				
	C. difficile		No antigen control	
	Spore Ab	CD1021 peptide 1 Ab	Spore Ab	CD1021 Ab1
Coupon 1	1.35	1.12	0.15	0.18
Coupon 2	1.64	1.24	0.18	0.21
Coupon 3	1.22	0.93	0.14	0.2

Example 12

Antigen affinity purification of antibodies

The purification of an antibody specific for a particular antigen and free of cross reactants from other immunoglobulins is often beneficial. Any of the polyclonal antibodies described in the above examples may be purified by affinity chromatography using the peptide or protein antigen covalently bound to an affinity matrix through NH_2 linkages. Purification can be achieved using peptide/protein bound affinity matrix such as Affygel (Biorad, Hercules, CA), AminoLink resin (Pierce, Rockford, IL) or CNBr activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ) in a column. The antibodies that are specific to the antigen bind to the column. The unbound antibodies and other serum proteins pass through the column. The antigen bound antibodies are then eluted from the column. The resulting purified antibody is highly specific.

Example 13

Antibodies to additional regions of *C. difficile* hypothetical protein CD1021

Following the procedures described in more detail in Example 4, murine polyclonal and monoclonal antibodies can be developed to additional polypeptide sequences of the hypothetical protein CD1021 *C. difficile* strain 630. For example, a polypeptide having residues 30 to 120 of the hypothetical protein CD1021 *C. difficile* strain 630 having the amino acid sequence of KSVDLYSDVY IEKYFNRDKV MEVNIEIDES DLKDMNENAI KEEFKVAKVT VDGDTYGNVG IRTKGNSSLI SVANSDSDRY SYKINFDKYN T (SEQ ID NO: 9) or a polypeptide having residues 194 to 293 of the hypothetical protein CD1021 *C. difficile* strain 630 having the amino acid sequence of VTGDLYKSDE GSSLQYKGDD PESYSNLIVE SDKKTADWSK

ITKLLKSLDT GEDIEKYLDV DSVLKNIAIN TALLNLDSYQ GSFAHNYYLY EQDGVFSMLP (SEQ ID NO: 10) can be used as immunogens. Polyclonal and monoclonal antibodies can also be developed to fragments thereof, including, for example, fragments of about 10-20 amino acids and about 14 amino acids of SEQ ID NO:9 and SEQ ID NO:10.

Example 14

Monoclonal antibodies

Monoclonal antibodies that bind to *C. difficile* endospores may be produced by a variety of known methods. Monoclonal antibodies may be produced using any of SEQ ID NO:1-12, 16-23, 38, and 45-50, and fragments thereof, as an antigen. For example, mice that have been immunized with the hypothetical CD1021 protein sequence SEQ ID NO:2, as described in Example 4, or the putative amidase protein sequence SEQ ID NO:6, as described in Example 6, may be used for the generation of murine monoclonal antibodies. Further, rabbits that have been immunized with the hypothetical CD1021 peptides SEQ ID NO:3 or SEQ ID NO:4, as described in Example 5, or the putative amidase peptide sequences SEQ ID NO:7 or SEQ ID NO:8, as described in Example 7, may be used for the generation of rabbit monoclonal antibodies. Epitomic's proprietary method for making monoclonal antibodies from rabbits rather than the conventional method of starting with mice may be used. The basic principal for making the antibody is the same as for mouse monoclonals. A proprietary rabbit fusion partner is used that can fuse to rabbit B-cells to create the rabbit hybridoma cells. Hybridomas are then screened to select for clones with specific and sensitive antigen recognition and the antibodies are characterized using a variety of methods.

Example 15

Blast analysis

The *C. difficile* strain 630 protein sequences for the hypothetical protein CD1021 (YP_001087502) (SEQ ID NO:1) and the putative N-acetylmuramoyl-L-alanine amidase cell surface protein (YP_001087517) (SEQ ID NO:5) were searched

for homologies against the approximately 988 microbial genomic sequences available at GenBank, RefSeq Nucleotides, EMBL, DDBJ, and PDB sequences (excluding HTGS0,1,2, EST, GSS, STS, PAT, WGS). Sequences producing significant alignments (E value of 0.0) were found only in the genomic sequence databases for *C. difficile* strain 630, *C. difficile* QCD-66c26, and *C. difficile* QCD-32g58.

Hypothetical protein CD1021 in *C. difficile* QCD-32g58.

For hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:1) the following sequences with significant homology (E value of 0.0) were found in *C. difficile* QCD-32g58.

GenBank Accession No. ZP_01804840, hypothetical protein CdifQ_04001048 in *C. difficile* QCD-32g58 having the amino acid sequence:

MIIFLCAVVG VYSTSSNKSV DLYSDVYIEK YFNRDKVMEV NIEIDESDLK
DMNENAIKEE FKVAKVTVDG DTYGNVGIRT KGNSSLTSVA NSDSDRYSYK
INFDKYNTSQ SMEGLTQLNL NNCYSDPSYM REFLTYSICE EMGLATPEFA
YAKVSINGEY HGLYLAVEGL KESYLENNFG NVTGDLYKSD EGSSLQYKGD
DPESYSNLIV ESDKKTADWS KITKLLKSLD TGEDIEKYLD VDSVLKNIAI
NTALLNLDSY QGSFAHNYYL YEQDGVFSML PWDFNMSFGG FSGFGGGSQS
IAIDEPTTGN LEDRPLISSL LKK (SEQ ID NO:11).

GenBank Accession No. ZP_01804841, hypothetical protein CdifQ_04001049 in *C. difficile* QCD-32g58 having the amino acid sequence:

MTTKLHDMIA SYVKEDPTAF YTYEEFEKNI TSSIEDSSDN KGFGNKGFDN
NNSNNSDSNN NSNSENKRSG NQSDKKEVNA ELTSSVVKTN TDNETENKTT
NDSESKNNTD KDKSGNDNNQ KLEGPRGKGG KSIPGVLEVA EDMSKTIKSQ
LSGETSSTKQ NSGDESSSGI KGSEKFDEDM SGMPEPPEGM DGKMPPGMGN
MDKGDMNGKN GNMNMDRNQD NPREAGGFGN RGGGSVSKTT TYFKLILGGA
SMIIMSIMLV GVSRVKRRRF IKSK (SEQ ID NO:12).

REGION 461827 to 462825 of GenBank Accession No. NZ_AAML04000007; *C. difficile* QCD-32g58 C_difficile_bld4_cont00007 having the nucleotide sequence:

AAGATAAAAA AATTTACCCT TCTTATCTCT ATTATGATTA TATTTTTATG
TGCTGTAGTT GGAGTTTATA GTACATCTAG CAACAAAAGT GTTGATTTAT
ATAGTGATGT ATATATTGAA AAATATTTTA ACAGAGACAA GGTTATGGAA
GTTAATATAG AGATAGATGA AAGTGACTTG AAGGATATGA ATGAAAATGC

TATAAAAGAA GAATTTAAGG TTGCAAAAGT AACTGTAGAT GGAGATACAT
ATGGAAACGT AGGTATAAGA ACTAAAGGAA ATTCAAGTCT TACATCTGTA
GCAAATAGTG ATAGTGATAG ATACAGCTAT AAGATTAATT TTGATAAGTA
TAATACTAGT CAAAGTATGG AAGGGCTTAC TCAATTAAAT CTTAATAACT
GTTACTCTGA CCCATCTTAT ATGAGAGAGT TTTTAACATA TAGTATTTGC
GAGGAAATGG GATTAGCGAC TCCAGAATTT GCATATGCTA AAGTCTCTAT
AAATGGCGAA TATCATGGTT TGTATTTGGC AGTAGAAGGA TTAAAAGAGT
CTTATCTTGA AAATAATTTT GGTAATGTAA CTGGAGACTT ATATAAGTCA
GATGAAGGAA GCTCGTTGCA ATATAAAGGA GATGACCCAG AAAGTTACTC
AAACTTAATC GTTGAAAGTG ATAAAAAGAC AGCTGATTGG TCTAAAATTA
CAAAACTATT AAAATCTTTG GATACAGGTG AAGATATTGA AAAATATCTT
GATGTAGATT CTGTCCTTAA AAATATAGCA ATAAATACAG CTTTATTAAA
CCTTGATAGC TATCAAGGCA GTTTTGCCCA TAACTATTAT TTATATGAGC
AAGATGGAGT ATTTTCTATG TTACCATGGG ATTTTAATAT GTCATTTGGT
GGATTTAGTG GTTTTGGTGG AGGTAGTCAA TCTATAGCAA TTGATGAACC
TACGACAGGT AATTTAGAAG ACAGACCTCT CATATCCTCG TTATTAAAA

(SEQ ID NO:13).

An amino acid sequence encoded by nucleotide sequence SEQ ID NO:13 is:

KIKKFTLLIS IMIIFLCAVV GVYSTSSNKS VDLYSDVYIE KYFNRDKVME
VNIEIDESDL KDMNENAIKE EFKVAKVTVD GDTYGNVGIR TKGNSSLTSV
ANSDSDRYSY KINFDKYNTS QSMEGLTQLN LNNCYSDPSY MREFLTYSIC
EEMGLATPEF AYAKVSINGE YHGLYLAVEG LKESYLENNF GNVTGDLYKS
DEGSSLQYKG DDPESYSNLI VESDKKTADW SKITKLLKSL DTGEDIEKYL
DVDSVLKNIA INTALLNLDS YQGSFAHNYY LYEQDGVFSM LPWDFNMSFG
GFSGFGGGSQ SIAIDEPTTG NLEDRPLISS LLK (SEQ ID NO:21).

And, REGION 462824 to 463732 of GenBank Accession No. NZ_AAML04000007; *C. difficile* QCD-32g58_difficile_bld4_cont00007 having the nucleotide sequence:

AAAAATGAGA CACACAAAAC AAAATACCAT AAATATCTGG AAGAGATAGT
AACAAAATAC CTAGATTCAG ACTATTTAGA GAATATGACA ACAAAATTGC
ATGACATGAT AGCATCATAT GTAAAAGAAG ACCCAACAGC ATTTTATACT
TATGAAGAAT TTGAAAAAAA TATAACATCT TCAATTGAAG ATTCTAGTGA
TAATAAGGGA TTTGGTAATA AAGGGTTTGA CAACAATAAC TCTAATAACA

GTGATTCTAA TAATAATTCT AATAGTGAAA ATAAGCGCTC TGGAAATCAA
AGTGATAAAA AAGAAGTTAA TGCTGAATTA ACATCAAGCG TAGTCAAAAC
TAATACAGAT AATGAAACTG AAAATAAAAC TACAAATGAT AGCGAAAGTA
AGAATAATAC AGATAAAGAT AAAAGTGGAA ATGATAATAA TCAAAAGCTA
GAAGGTCCTA GGGGTAAAGG AGGTAAGTCA ATACCAGGGG TTTTGGAAGT
TGCAGAAGAT ATGAGTAAAA CTATAAAATC TCAATTAAGT GGAGAAACTT
CTTCGACAAA GCAAAACTCT GGTGATGAAA GTTCAAGTGG AATTAAAGGT
AGTGAAAAGT TTGATGAGGA TATGAGTGGT ATGCCAGAAC CACCTGAGGG
AATGGATGGT AAAATGCCAC CAGGAATGGG TAATATGGAT AAGGGAGATA
TGAATGGTAA AAATGGCAAT ATGAATATGG ATAGAAATCA AGATAATCCA
AGAGAAGCTG GAGGTTTTGG CAATAGAGGA GGAGGCTCTG TGAGTAAAAC
AACAACATAC TTCAAATTAA TTTTAGGTGG AGCTTCAATG ATAATAATGT
CGATTATGTT AGTAGGTGTA TCAAGGGTAA AGAGAAGAAG ATTTATAAAG
TCAAAATAA (SEQ ID NO:14).

An amino acid sequence encoded by nucleotide sequence SEQ ID NO:14 is:

KNETHKTKYH KYLEEIVTKY LDSDYLENMT TKLHDMIASY VKEDPTAFYT
YEEFEKNITS SIEDSSDNKG FGNKGFDNNN SNNSDSNNNS NSENKRSGNQ
SDKKEVNAEL TSSVVKTNTD NETENKTTND SESKNNTDKD KSGNDNNQKL
EGPRGKGGKS IPGVLEVAED MSKTIKSQLS GETSSTKQNS GDESSSGIKG
SEKFDEDMSG MPEPPEGMDG KMPPGMGNMD KGDMNGKNGN MNMDRNQDNP
REAGGFGNRG GGSVSKTTTY FKLILGGASM IIMSIMLVGV SRVKRRRFIK
SK (SEQ ID NO:22).

Hypothetical protein CD1021 in *C. difficile* QCD-66c26

For hypothetical protein CD1021 of *C. difficile* strain 630 (SEQ ID NO:1) one sequence with significant homology (E value of 0.0) was found in *C. difficile* QCD-66c26.

The complement of REGION 15690 to 17597 of GenBank Accession No. NZ_ABFD01000037; *C. difficile* QCD-66c26 contig00122 having the nucleotide sequence:

ATGAAAGATA AAAAATTTAC CCTTCTTATC TCTATTATGA TTATATTTTT
ATGTGCTGTA TTGGAGTTT ATAGTACATC TAGCAACAAA AGTGTTGATT
TATATAGTGA TGTATATATT GAAAAATATT TTAACAGAGA CAAGGTTATG

GAAGTTAATA TAGAGATAGA TGAAAGTGAC TTGAAGGATA TGAATGAAAA
TGCTATAAAA GAAGAATTTA AGGTTGCAAA AGTAACTGTA GATGGAGATA
CATATGGAAA CGTAGGTATA AGAACTAAAG GAAATTCAAG TCTTACATCT
GTAGCAAATA GTGATAGTGA TAGATACAGC TATAAGATTA ATTTTGATAA
GTATAATACT AGTCAAAGTA TGGAAGGGCT TACTCAATTA AATCTTAATA
ACTGTTACTC TGACCCATCT TATATGAGAG AGTTTTTAAC ATATAGTATT
TGCGAGGAAA TGGGATTAGC GACTCCAGAA TTTGCATATG CTAAAGTCTC
TATAAATGGC GAATATCATG GTTTGTATTT GGCAGTAGAA GGATTAAAAG
AGTCTTATCT TGAAAATAAT TTTGGTAATG TAACTGGAGA CTTATATAAG
TCAGATGAAG GAAGCTCGTT GCAATATAAA GGAGATGACC CAGAAAGTTA
CTCAAACTTA ATCGTTGAAA GTGATAAAAA GACAGCTGAT TGGTCTAAAA
TTACAAAACT ATTAAAATCT TTGGATACAG GTGAAGATAT TGAAAAATAT
CTTGATGTAG ATTCTGTCCT TAAAAATATA GCAATAAATA CAGCTTTATT
AAACCTTGAT AGCTATCAAG GCAGTTTTGC CCATAACTAT TATTTATATG
AGCAAGATGG AGTATTTTCT ATGTTACCAT GGGATTTTAA TATGTCATTT
GGTGGATTTA GTGGTTTTGG TGGAGGTAGT CAATCTATAG CAATTGATGA
ACCTACGACA GGTAATTTAG AAGACAGACC TCTCATATCC TCGTTATTAA
AAAATGAGAC ACACAAAACA AAATACCATA AATATCTGGA AGAGATAGTA
ACAAAATACC TAGATTCAGA CTATTTAGAG AATATGACAA CAAAATTGCA
TGACATGATA GCATCATATG TAAAAGAAGA CCCAACAGCA TTTTATACTT
ATGAAGAATT TGAAAAAAAT ATAACATCTT CAATTGAAGA TTCTAGTGAT
AATAAGGGAT TTGGTAATAA AGGGTTTGAC AACAATAACT CTAATAACAG
TGATTCTAAT AATAATTCTA ATAGTGAAAA TAAGCGCTCT GGAAATCAAA
GTGATAAAAA AGAAGTTAAT GCTGAATTAA CATCAAGCGT AGTCAAAACT
AATACAGATA ATGAAACTGA AAATAAAACT ACAAATGATA GCGAAAGTAA
GAATAATACA GATAAAGATA AAAGTGGAAA TGATAATAAT CAAAAGCTAG
AAGGTCCTAG GGGTAAAGGA GGTAAGTCAA TACCAGGGGT TTTGGAAGTT
GCAGAAGATA TGAGTAAAAC TATAAAATCT CAATTAAGTG GAGAAACTTC
TTCGACAAAG CAAAACTCTG GTGATGAAAG TTCAAGTGGA ATTAAAGGTA
GTGAAAAGTT TGATGAGGAT ATGAGTGGTA TGCCAGAACC ACCTGAGGGA
ATGGATGGTA AAATGCCACC AGGAATGGGT AATATGGATA AGGGAGATAT
GAATGGTAAA AATGGCAATA TGAATATGGA TAGAAATCAA GATAATCCAA
GAGAAGCTGG AGGTTTTGGC AATAGAGGAG GAGGCTCTGT GAGTAAAACA
ACAACATACT TCAAATTAAT TTTAGGTGGA GCTTCAATGA TAATAATGTC

GATTATGTTA GTAGGTGTAT CAAGGGTAAA GAGAAGAAGA TTTATAAAGT CAAAATAA (SEQ ID NO:15).

An amino acid sequence encoded by nucleotide sequence SEQ ID NO:15 is:

MKDKKFTLLI SIMIIFLCAV VGVYSTSSNK SVDLYSDVYI EKYFNRDKVM EVNIEIDESD LKDMNENAIK EEFKVAKVTV DGDTYGNVGI RTKGNSSLTS VANSDSDRYS YKINFDKYNT SQSMEGLTQL NLNNCYSDPS YMREFLTYSI CEEMGLATPE FAYAKVSING EYHGLYLAVE GLKESYLENN FGNVTGDLYK SDEGSSLQYK GDDPESYSNL IVESDKKTAD WSKITKLLKS LDTGEDIEKY LDVDSVLKNI AINTALLNLD SYQGSFAHNY YLYEQDGVFS MLPWDFNMSF GGFSGFGGGS QSIAIDEPTT GNLEDRPLIS SLLKNETHKT KYHKYLEEIV TKYLDSDYLE NMTTKLHDMI ASYVKEDPTA FYTYEEFEKN ITSSIEDSSD NKGFGNKGFD NNNSNNSDSN NNSNSENKRS GNQSDKKEVN AELTSSVVKT NTDNETENKT TNDSESKNNT DKDKSGNDNN QKLEGPRGKG GKSIPGVLEV AEDMSKTIKS QLSGETSSTK QNSGDESSSG IKGSEKFDED MSGMPEPPEG MDGKMPPGMG NMDKGDMNGK NGNMNMDRNQ DNPREAGGFG NRGGGSVSKT TTYFKLILGG ASMIIMSIML VGVSRVKRRR FIKSK (SEQ ID NO:23).

Fig. 1 demonstrates the high degree of homology between the amino acid sequences for hypothetical CD1021 proteins YP_001087502 from *C. difficile* strain 630 (SEQ IDNO:1), ZP_01804840, from *C. difficile* QCD-32g58 (SEQ ID NO:11), ZP_01804841 from *C. difficile* QCD-32g58 (SEQ ID NO:12), Region 461827 to 462825 NZ_AAML04000007 from *C. difficile* QCD-32g58 (SEQ ID NO:21), Region 462824 to 463732 of NZ_AAML04000007 *C. difficile* QCD-32g58 (SEQ ID NO:22), and the complement of Region 15690 to 17597 NZ-ABFD01000037 from *C. difficile* QCD-66c26 (SEQ ID NO:23). A consensus sequence is shown as SEQ ID NO:38.

Putative N-acetylmuramoyl-L-alanine amidase cell surface proteins

For putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* strain 630 (SEQ ID NO:5) the following additional sequences with significant homology (E value of 0.0) in *C. difficile* strain 630 were found.

GenBank Accession No. YP_001087516, cell surface protein putative N-acetylmuramoyl-L-alanine amidase, *C. difficile* strain 630, having the amino acid sequence:

MLSKEINMRR NTKLLTTGIL SMAIVTPTMA FATESNAMEN NADLNINLEK
KSIVLGSTSK VSVKFKEKPD ADSITLKYKC YDMPLDTTLN YNQSTESYEG
TINYNKDPEY LNVWELQGIT INSKNNPKTL NKQELEKMGL NLKDYNVTQE
CIIEDITSRK DVNKYLRKTS APITELTGSD RYETAVKISK EGWKNGSDKV
VIINGDVSID GIISTPLATT YNAPILLVEK NNVPNSVKSE LKRLNPRDVI
IIGDENAISK TTANQIKSTV NASQTRLKGS NRYETSLLIA KEIDKNHDVE
KVYITNANGG EVDALTIAAK AGQDKQPIIL TDKNSITDNT YKWLKSEDLQ
NAYFIGGPQM ISTNVINKVN DITKDNVTNN RVYGADRHET NANVIKKFYT
DDELEAVLVA KSDVLVDALA AGPLAANLKS PILITPKTYV SAYHKENLEA
KSANKVYKIG GGLTSKVMSS IASSLSKHNT TPTEPGNSGG KTVMIDPGHG
GSDTGTTGKP LGGIREKDYT LNTSLATTEY LRSKGFNVIM TRDTDKTLSL
GNRTALSNSL RPDLFTSIHY NASDTTGNGV EVFYKLKDKD GGTTKTVATN
ILNRILEKFN LKNRGAKTRT LSTDPTKDYL YVLRNNDMPA VLVECAFLDN
EKDMSLLNTS NKVKEMGTQI GKGIEDSLK (SEQ ID NO:16).

And, GenBank Accession No. YP_001089297 (cell surface protein *C. difficile* 630) having the amino acid sequence:

MMKKTTKLLA TGMLSVAMVA PNVALAAENT TANTESNSDI NINLQRKSVV
LGSKSNASVK FKEKLNADSI TLNFMCYDMP LEATLNYNEK TDSYEGVINY
NKDPEYLNVW ELQSIKINGK DEQKVLNKED LESMGLNLKD YDVTQEFIIS
DANSTKAVNE YMRKTSAPVK KLAGATRFET AVEISKQGWK DGSSKVVIVN
GELAADGITA TPLASTYDAP ILLANKDDIP ESTKAELKRLNPSDVIIIGD
DGSVSQKAVS QIKSAVNVNV TRIGGVDRHE TSLLIAKEID KYHDVNKIYI
ANGYAGEYDA LNISSKAGED QQPIILANKD SVPQGTYNWL SSQGLEEAYY
IGGSQSLSSK IIDQISKIAK NGTSKNRVSG ADRHETNANV IKTFYPDKEL
SAMLVAKSDI IVDSITAGPL AAKLKAPILI TPKTYVSAYH STNLSEKTAE
TVYQIGDGMK DSVINSIASS LSKHNAPTEP DNSGSAAGKT VVIDPGHGGS
DSGATSGLNG GAQEKKYTLN TALATTEYLR SKGINVVMTR DTDKTMALGE
RTALSNTIKP DLFTSIHYNA SNGSGNGVEI YYKVKDKNGG TTKTAASNIL
KRILEKFNMK NRGIKTRTLD NGKDYLYVLR NNNYPAILVE CAFIDNKSDM
DKLNTAEKVK TMGTQIGIGI EDTVK (SEQ ID NO:17).

For putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* strain 630 (SEQ ID NO:5) the following sequences with significant homology (E value of 0.0) were found in *C. difficile* QCD-32g58.

GenBank Accession No. ZP_01804350 (hypothetical protein CdifQ_04001133; *C. difficile* QCD-32g58) having the amino acid sequence:

MFRFKEKPDA DSITLKYKCY DMPLDTTLNY NQSTESYEGT INYNKDPEYL
NVWELQGITI NSKNNPKTLN KQELEKMGLN LKDYNVTQEC IIEDITSRKD
VNKYLRKTSA PITELTGSDR YETAVKISKE GWKNGSDKVV IINGDVSIDG
IISTPLATTY NAPILLVEKN NVPNSVKSEL KRLNPRDVII IGDENAISKT
TANQIKSTVN ASQTRLKGSN RYETSLLIAK EIDKNHDVEK VYITNANGGE
VDALTIAAKA GQDKQPIILT DKNSITDNTY KWLKSEDLQN AYFIGGPQMI
STNVINKVND ITKDNVTNNR VYGADRHETN ANVIKKFYTD DELEAVLVAK
SDVLVDALAA GPLAANLKSP ILITPKTYVS AYHKDNLEAK SANKVYKIGG
GLTSKVMNSI ASSLSKHNTT PTEPGNSGGK TVMIDPGHGG SDTGTTGKPL
GGIKEKDYTL NTSLATTEYL RSKGFNVIMT RDTDKTLSLG NRTALSNSLR
PDLFTSIHYN ASDTTGNGVE VFYKLKDKDG GTTKTVATNI LNRILEKFNL
KNRGAKTRTL STDPTKDYLY VLRNNDMPAV LVECAFLDNE KDMSLLNTSN
KVKEMGTQIG KGIEDSLK (SEQ ID NO:18).

GENEBANK Accession No. ZP_01804351 (hypothetical protein CdifQ_04001134; *C. difficile* QCD-32g58) having the amino acid sequence:

MLSKEINMRR NTKLLTTGIL SMAIVAPTMA FATESNAMEN NADLNINLEK
KSIVLGSKSK VSVKFKEKPD ADSITLKYKC YDMPLDTTLN YNQSTGAYEG
TINYNQDPEY LNVWELQGIT INSKNNPKTL NGQDLEKMGL NLKDYNVTQE
CIIEDITSRK DVNKYLRKTS APITELTGSD RYETAVKISK EGWKNGSDKV
VIINGDVSID GIISTPLATT YNAPILLVEK NNVPNSVKSE LKRLNPKDII
IIGDENAISK TTANQIKSTV NASQTRLNGS NRYETSLLIA KEIDKNHDVE
KVYITNANGG EVDALTIAAK AGQDKQPIIL TDKDSITDNT YKWLKSEDLQ
NAYFIGGPQM ISTNVINKVN GITKDSVTNN RVYGADRHET NANVIKKFYT
EDEIEAVLVA KSDVLVDALA AGPLAANLKS PILITPKTYV SAYHKDNLEA
KSANKVYKIG GGLTSKVMSS IASSLSKHNT TPTEPGNSGG KTVMIDPGHG
GSAPGNSSGG MIEKDYNLNT SLATTEYLRS KGFNVIMTRD TDKTLSLGNR
TA (SEQ ID NO:19).

And, GenBank Accession No. ZP_01802273 (hypothetical protein CdifQ_04003247; *C. difficile* QCD-32g58) having the amino acid sequence:

MMKKTTKLLA TGMLSVAMVA PNVALAAENT TANTESNSDI NINLQRKSVV
LGSKSNASVK FKEKLNADSI TLNFMCYDMP LEATLNYNEK TDSYEGVINY

NKDPEYLNVW ELQSIKINGK DEQKVLNKED LESMGLNLKD YDVTQEFIIS
DANSTKAVNE YMRKTSAPVK KLAGATRFET AVEISKQGWK DGSSKVVIVN
GELAADGITA TPLASTYDAP ILLANKDDIP ESTKAELKRL NPSDVIIIGD
DGSVSQKAVS QIKSAVNVNV TRIGGVDRHE TSLLIAKEID KYHDVNKIYI
ANGYAGEYDA LNISSKAGED QQPIILANKD SVPQGTYNWL SSQGLEEAYY
IGGSQSLSSK IIDQISKIAK NGTSKNRVSG ADRHETNANV IKTFYPDKEL
SAMLVAKSDI IVDSITAGPL AAKLKAPILI TPKTYVSAYH STNLSEKTAG
TVYQIGDGMK DSVINSIASS LSKHNAPTEP DNSGSAAGKT VVIDPGHGGS
DSGATSGLNG GAQEKKYTLN TALATTEYLR SKGINVVMTR DTDKTMALGE
RTALSNTIKP DLFTSIHYNA SNGAGNGVEI YYKVKDKNGG TTKTAASNIL
KRILEKFNMK NRGIKTRTLD NGKDYLYVLR NNNYPAILVE CAFIDNKSDM
DKLNTAEKVK TMGTQIGIGI EDTVK (SEQ ID NO:20).

The antibodies described herein may bind to one or more of the amino acid sequences of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23, and fragments thereof. Antibodies described herein may bind to one or more of the amino acid sequences encoded by the genomic nucleotide sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15 and polypeptide fragment thereof.

Antibodies may be produced, by any of a variety methods, including, but not limited to, any of those described herein, that bind to one or more of the amino acid sequences of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23, and fragment thereof. Antibodies may be produced, by any of the various methods described herein, that bind to one or more of the amino acid sequences encoded by the genomic nucleotide sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15 and polypeptide fragment thereof. Any such antibodies may be used in methods of detecting *C. difficile* spores in a sample.

Example 16

Cloning and sequencing CD1021 from *C. difficile* ATCC 9689

A CD1021 coding sequence was amplified by PCR using a forward primer with NheI site (5'-TAAGCTAGCATGAAAGATAAAAAATTTACC-3') (SEQ ID NO:24),

a reverse primer with XhoI site (5'-TTACTCGAGTTTTGACTTTATAAATCTTCT-3') (SEQ ID NO:25) and genomic DNA from ATCC strain 9689 as the template. A stop codon was removed from the reverse primer to facilitate addition of 6-HIS tag to the end of the sequence. The resulting fragment size was 1923 base pairs (bp).

Similarly, the region corresponding to amino acid residues 30 to 120 of CD1021 in *C. difficile* strain 630 (SEQ ID NO:9) (also referred to herein as "fragment 1") was amplified using genomic DNA from ATCC strain 9689 as the template with a forward primer with NheI site (5'-ACAGCTAGCATGAAAAGTGTTGATTTATATAGT-3') (SEQ ID NO:26) and a reverse primer with XhoI site (5'-ACTCTCGAGAGTATTATAC TTATCAAAATTA-3') (SEQ ID NO:27). The resulting fragment size was 294 bp and included an ATG initiation codon.

The region corresponding to amino acid residues 194 to 293 of CD1021 in *C. difficile* strain 630 (SEQ ID NO:10) (also referred to herein as "fragment 2") was amplified using genomic DNA from ATCC strain 9689 as the template with a forward primer with NheI site (5'-AATGCTAGCATGGTAACTGGAG ACTTATATAAGTCA-3') (SEQ ID NO:28) and a reverse primer with XhoI site (5'-AAACTCGAGTGG TAACA TAGAAAATACTCCAT-3') (SEQ ID NO:29). The resulting fragment size was 321 bp and included an ATG initiation codon.

And, the region corresponding to amino acid residues 505 to 604 of CD1021 in *C. difficile* strain 630 (SEQ ID NO:2) (also referred to herein as "fragment 3") was amplified using genomic DNA from ATCC strain 9689 as the template with a forward primer with NheI site (5'-GCAGCTAGCATGAGTAAAACTATAAAATCTCAA-3') (SEQ ID NO:30) and a reverse primer with XhoI site (5'-AATCTCGAGGAAGTATGTTGTTGTTTTACT CAC-3') (SEQ ID NO:31). The resulting fragment size was 321 bp and included an ATG initiation codon.

The resultant PCR reactions were run on an agarose gel (0.8%) and products of the expected size were observed. The PCR fragments were cloned using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The transformed colonies were picked and grown in LB with Kanamycin (100 μg/ml) for 16 hours at 37° C. The plasmid was isolated from these cultures using Qiaprep spin miniprep kit (Qiagen, Valencia, CA) and the plasmids were cut with EcoRI (Invitrogen) and analyzed by agarose gel. The clones having the insert were sequenced using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied

Biosystems, Foster City, CA) with M13 forward -20 primer (5'-GTAAAACGACGGCCAGT-3') (SEDQ ID NO:32) and M13 reverse -27 primer (5'-CAGGAAACAGCTATGAC-3') (SEQ ID NO:33). Appropriate internal primers were used to obtain the complete sequence of the 1923 bp CD1021 coding sequence. Three clones were selected for further characterization for each of the four cloning reactions. For the 1923 bp CD1021 coding sequence, the three clones were pCD1021-1, pCD1021-2, and pCD1021-3. For fragment 1, the three clones were pCD1021-Fr1-1, pCD1021-Fr1-2, and pCD1021-Fr1-3. For fragment 2, the three clones were pCD1021-Fr2-1, pCD1021-Fr2-2, and pCD1021-Fr2-3. For fragment 3, the three clones were pCD1021-F3-1, pCD1021-Fr3-2, and pCD1021-Fr3-3.

The nucleotide sequence of *C. difficile* ATCC 9689 CD1021 fragment in plasmid pCD1021-2 is:

ATGAAAGATA AAAAATTTAC CCTTCTTATC TCGATTATGA TTATATTTTT
ATGTGCTGTA GTTGGAGTTT ATAGTACATC TAGCAACAAA AGTGTTGATT
TATATAGTGA TGTATATATT GAAAAATATT TTAACAGAGA CAAGGTTATG
GAAGTTAATA TAGAGATAGA TGAAAGTGAC TTGAAGGATA TGAATGAAAA
TGCTATAAAA GAAGAATTTA AGGTTGCAAA AGTAACTGTA GATGGAGATA
CATATGGAAA CGTAGGTATA AGAACTAAAG GAAATTCAAG TCTTATATCT
GTAGCAAATA GTGATAGTGA TAGATACAGC TATAAGATTA ATTTTGATAA
GTATAATACT AGTCAAAGTA TGGAAGGGCT TACTCAATTA AATCTTAATA
ACTGTTACTC TGACCCATCT TATATGAGAG AGTTTTTAAC ATATAGTATT
TGCGAGGAAA TGGGATTAGC GACTCCAGAA TTTGCATATG CTAAAGTCTC
TATAAATGGC GAATATCATG GTTTGTATTT GGCAGTAGAA GGATTAAAAG
AGTCTTATCT TGAAAATAAT TTTGGTAATG TAACTGGAGA CTTATATAAG
TCAGATGAAG GAAGCTCGTT GCAATATAAA GGAGATGACC CAGAAAGTTA
CTCAAACTTA ATCGTTGAAA GTGATAAAAA GACAGCTGAT TGGTCTAAAA
TCACAAAACT ATTAAAATCT TTGGATACAG GTGAAGATAT TGAAAAATAT
CTTGATGTAG ATTCTGTCCT TAAAAATATA GCAATAAATA CAGCTTTATT
AAACCTTGAT AGCTATCAAG GGAGTTTTGC CCATAACTAT TATTTATATG
AGCAAGATGG AGTATTTTCT ATGTTACCAT GGGATTTTAA TATGTCATTT
GGTGGATTTA GTGGTTTTGG TGGAGGTAGT CAATCTATAG CAATTGATGA
ACCTACGACA GGTAATTTAG AAGACAGACC TCTCATATCC TCGTTATTAA
AAAATGAGAC ATACAAAACA AAATACCATA AATATCTGGA AGAGATAGTA

ACAAAATACC TAGATTCAGA CTATTTAGAG AATATGACAA CAAAATTGCA
TGACATGATA GCATCATATG TAAAAGAAGA CCCAACAGCA TTTTATACTT
ATGAAGAATT TGAAAAAAAT ATAACATCTT CAATTGAAGA TTCTAGTGAT
AATAAGGGAT TTGGTAATAA AGGGTTTGAC AACAATAACT CTAATAACAG
TGATTCTAAT AATAATTCTA ATAGTGAAAA TAAGCGCTCT GGAAATCAAA
GTGATGAAAA AGAAGTTAAT GCTGAATTAA CATCAAGCGT AGTCAAAGCT
AATACAGATA ATGAAACTAA AAATAAAACT ACAAATGATA GTGAAAGTAA
GAATAATACA GATAAAGATA AAAGTGGAAA TGATAATAAT CAAAAGCTAG
AAGGTCCTAT GGGTAAAGGA GGTAAGTCAA TACCAGGGGT TTTGGAAGTT
GCAGAAGATA TGAGTAAAAC TATAAAATCT CAATTAAGTG GAGAAACTTC
TTCGACAAAG CAAAACTCTG GTGATGAAAG TTCAAGTGGA ATTAAAGGTA
GTGAAAAGTT TGATGAGGAT ATGAGTGGTA TGCCAGAACC ACCTGAGGGA
ATGGATGGTA AAATGCCACC AGGAATGGGT AATATGGATA AGGGAGATAT
GAATGGTAAA AATGGCAATA TGAATATGGA TAGAAATCAA GATAATCCAA
GAGAAGCTGG AGGTTTTGGC AATAGAGGAG GAGGCTCTGT GAGTAAAACA
ACAACATACT TCAAATTAAT TTTAGGTGGA GCTTCAATGA TAATAATGTC
GATTATGTTA GTTGGTGTAT CAAGGGTAAA GAGAAGAAGA TTTATAAAGT
CAAAA (SEQ ID NO:39). The translated amino acid sequence of CD1021 fragment in plasmid pCD1021-2 is:

MKDKKFTLLISIMIIFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDE
SDLKDMNENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLISVANSDSDRYSYKINFD
KYNTSQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHG
LYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWS
KITKLLKSLDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFS
MLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLKNETYKTKYHKYLEE
IVTKYLDSDYLENMTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGN
KGFDNNNSNNSDSNNNSNSENKRSGNQSDEKEVNAELTSSVVKANTDNETKNKTTNDS
ESKNNTDKDKSGNDNNQKLEGPMGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQN
SGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQ
DNPREAGGFGNRGGGSVSKTTTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK
(SEQ ID NO:45).

The nucleotide sequence of *C. difficile* ATCC 9689 CD1021 fragment (30 to 120 amino acid residues) in plasmid pCD1021-Fr1-1 is:

ATGAAAAGTG TTGATTTATA TAGTGATGTA TATATTGAAA AATATTTTAA
CAGAGACAAG GTTATGGAAG TTAATATAGA GATAGATGAA AGTGACTTGA
AGGATATGAA TGAAAATGCT ATAAAAGAAG AATTTAAGGT TGCAAAAGTA
ACTGTAGATG GAGATACATA TGGAAACGTA GGTATAAGAA CTAAAGGAAA
TTCAAGTCTT ATATCTGTAG CAAATAGTGA TAGTGATAGA TACAGCTATA
AGATTAATTT TGATAAGTAT AATACT (SEQ ID NO:40).

The translated amino acid sequence of CD1021 fragment (30 to 120 amino acid residues) in plasmid pCD1021-Fr1-1 is:
MKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMNENAIKEEFKVAKVTVDGDT
YGNVGIRTKGNSSLISVANSDSDRYSYKINFDKYNT (SEQ ID NO:46).

The nucleotide sequence of *C. difficile* ATCC 9689 CD1021 fragment (194 to 293 amino acid residues) in plasmid pCD1021-Fr2-1 is:
ATGGTAACTG GAGACTTATA TAAGTCAGAT GAAGGAAGCT CGTTGCAATA
TAAAGGAGAT GACCCAGAAA GTTACTCAAA CTTAATCGTT GAAAGTGATA
AAAAGACAGC TGATTGGTCT AAAATCACAA AACTATTAAA ATCTTTGGAT
ACAGGTGAAG ATATTGAAAA ATATCTTGAT GTAGATTCTG TCCTTAAAAA
TATAGCAATA AATACAGCTT TATTAAACCT TGATAGCTAT CAAGGGAGTT
TTGCCCATAA CTATTATTTA TATGAGCAaG ATGGAGTATT TTCTATGTTA
CCA (SEQ ID NO:41).

The translated amino acid sequence of CD1021 fragment (194 to 293 amino acid residues) in plasmid pCD1021-Fr2-1 is:
MVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKSLDTGEDIEK
YLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLP (SEQ ID NO:47).

The nucleotide sequence of *C. difficile* ATCC 9689 CD1021 fragment (505 to 604 amino acid residues) in plasmid pCD1021-Fr3-1 is:
ATGAGTAAAA CTATAAAATC TCAATTAAGT GGAGAAACTT CTTCGACAAA
GCAAAACTCT GGTGATGAAA GTTCAAGTGG AATTAAAGGT AGTGAAAAGT
TTGATGAGGA TATGAGTGGT ATGCCAGAAC CACCTGAGGG AATGGATGGT
AAAATGCCAC CAGGAATGGG TAATATGGAT AAGGGAGATA TGAATGGTAA
AAATGGCAAT ATGAATATGG ATAGAAATCA AGATAATCCA AGAGAAGCTG
GAGGTTTTGG CAATAGAGGA GGAGGCTCTG TGAGTAAAAC AACAACATAC
TTC (SEQ ID NO:42).

The translated amino acid sequence of CD1021 fragment (505 to 604 amino acid residues) in plasmid pCD1021-Fr3-1 is:
MSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPG
MGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKTTTYF (SEQ ID NO:48).

Example 17

Expression of *C. difficile* ATCC 9689 CD1021 clones

The recombinant plasmid pCD1021-2, obtained in Example 16, and the expression vector pET21-a(+) (Novagen, Madison, WI) were separately cut with the restriction enzymes NheI and XhoI (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. The restriction enzyme-digested pCD1021-2 DNA was run on an agarose gel (0.8%) and the full-length CD1021 fragment was purified from the gel using Qiaquick gel extraction kit (Qiagen). The resultant fragment (1917 bp) was ligated into the restriction enzyme-digested expression vector pET21-a(+) using T4 DNA ligase (Invitrogen) according to manufacturer's instructions.

The ligated mixture was used to transform TOP10 chemically competent *E. coli* cells and plated on LB Agar with ampicillin (50 μg/ml). The plates were incubated at 37° C for 12 to 16 hours and the recombinant clones were picked and grown in LB with ampicillin (100 μg/ml) for 16 hours at 37° C. The plasmid was isolated from these cultures by miniprep using alkaline lysis protocol (Miniprep kit, Qiagen) and the plasmids were cut with NheI and XhoI and analyzed by agarose gel. The clones having the full-length CD1021 insert were selected and tested for expression of proteins according to manufacturer's instruction.

Similarly, the fragment 1, fragment 2, and fragment 3 of clones CD1021-Fr1-1, CD1021-Fr2-2, and CD1021-Fr3-1 obtained in Example 16, were restriction digested with NheI and XhoI and cloned into NheI/XhoI restricted pET21a+. The recombinant clones were picked and analyzed as described above. The clones having the inserts were selected and tested for expression of proteins according to manufacturer's instruction.

The recombinant clones were transformed into competent cells of BLR(DE3) and plated on LB Agar with ampicillin (50 μg/ml). The plates were incubated at 37° C for

12 to 16 hours. Several colonies were picked and grown in 5 ml of LB with ampicillin (100 μg/ml) for 16 hours at 37° C. The overnight grown clones were diluted 1:100 into 5 ml LB with ampicillin (100 μg/ml) and grown to an OD600 of 0.6 to 0.7. The clones were induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO) for 3 hrs either at 37° C or 15° C using Lab-Line MaxQ 4000 Incubated and Refrigerated Shakers (Barnstead International, Dubuque, Iowa).

The induced cells were spun at 5000 rpm at 4° C for 10 min and resuspended in 1 ml of 0.1 mM TRIS-HCl buffer, pH 8.0. The cells were sonicated using Branson Digital Sonifier model S-250D (Branson, Danbury, CT) using 1/8 inch tapered microtip for 10 sec to lyse the cells. The cell extracts were analyzed by SDS-PAGE. The gels were stained with Coomassie blue and the induced cells showed better expression of recombinant protein of expected molecular weight at 15° C than at 37° C.

Example 18

Cloning and sequencing amidase (CD1036) from *C. difficile* ATCC 9689

A putative N-acetylmuramoyl-L-alanine amidase cell surface protein coding sequence was amplified by PCR using a forward primer with NcoI site (5'-AATCCATG G TAAGTAAGGAGATTAATATG-3') (SEQ ID NO:34), a reverse primer with XhoI site (5'-TTCCTCGAGTTTTAATGAATCTTCTATTCC-3') (SEQ ID NO:35) and genomic DNA from *C. difficile* ATCC strain 9689 as the template. A stop codon was removed from the reverse primer to facilitate addition of 6-HIS tag to the end of the sequence. The resulting fragment size was 2045 bp.

Similarly, the region corresponding to amino acid residues 294 to 393 of the putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* 630 (SEQ I DNO:6) was amplified using genomic DNA from ATCC strain 9689 as the template with a forward primer with NcoI site (5'-AGG CCA TGGATAAAAATCATGATGTGGAA-3') (SEQ ID NO:36) and a reverse primer with XhoI site (5'-TTTCTCGAGGTTTGCATTTG TTTCGTGTCT-3') (SEQ ID NO:37). The resulting fragment size was 317 bp and included an ATG initiation codon.

The resultant PCR fragments were cloned using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, San Diego, CA) and recombinant clones sequenced, using the

procedures described in Example 16. Three clones were characterized for each of the two cloning reactions. For the 2045 bp CD1036 coding sequence, the three clones were pCD1036-1, pCD1036-2, and pCD1036-3. For fragment 1, the three clones were pCD1036-Fr1-1, pCD1036-Fr1-2, and pCD1036-Fr1-3.

The nucleotide sequence of *C. difficile* ATCC 9689 CD1036 fragment in plasmid pCD1036-2 is:

ATGGTAAGTA AGGAGATTAA TATGAGAAGA AATACAAAAT TATTAACAAC
AGGGATTCTT TCAATGGCAA TCGTCGCACC TACAATGGCA TTTGCTACTG
AATCTAATGC TATGGAAAAT AACGCTGATT TAAATATAAA CTTAGAGAAA
AAAAGTATCG TTTTAGGTAG CAAATCAAAA GTTAGTGTCA AATTTAAAGA
AAAACCAGAT GCAGATAGCA TTAcATTAAA GTATAAATGC TATGACATGC
CATTGAATAC AACTCTAAAT TACAATCAAT CAACTGGGGC ATATGAAGGA
ACTATCAATT ATAACCAAGA CCCAGAATAT CTAAATGTTT GGGAACTACA
AGGGATAACA ATAAACAGCA AAAATAATCa TAAAACTTTA AACAGACAAG
ACCTAGAAAA GCTGGGATTA AATTTAAAAG ACTATAATGT AACACAGGAA
TGTATAATTG AAGATATAAC TTCTAGAAAA GATGTAAATA AATATTTGAG
AAAAACTTCT TCACCTATTA CAGAACTTAC AGGAAGTGAT AGATATGAAA
CAGCAGTTAA AATAAGTAAA GAGGGCTGGA AAAATGGTTC AGATAAGGTA
GTTATAATAA ATGGGGATGT AAGTATAGAT GGCATTATAT CAACTCCACT
GGCAACCACA TATAATGCAC CAATACTTTT GGTTGAAAAA AACAATGTAC
CTAATAGTGT AAAATCAGAA TTAAAGCGCC TAAACCCTAA AGATATAATT
ATAATTGGAG ATGAGAATGC TATTTCTAAA ACTACTGCTA ATCAAATTAA
ATCAACTGTA AATGCTAGTC AAACACGTTT AAATGGTTCT AATAGATATG
AGACATCTTT ATTGATAGCA AAGGAAATAG ATAAAAATCA TGATGTGGAA
AAAGTATACA TAACAAATGC TAATGGCGGA GAAGTGGATG CACTTACTAT
AGCAGCAAAA GCAGGTCAAG ACAAGCAACC AATTATATTA ACTGATAAAG
ATAGTATTAC AGACAATACA TATAAATGGT TAAAGAGTGA GGATTTACAA
AATGCTTATT TTATAGGTGG TCCTCAAATG ATATCAACAA ATGTTATAAA
TAAGGTAAAT GGAATAACTA AAGATAGTGT TACTAATAAT AGAGTATACG
GAGCAGATAG ACACGAAACA AATGCAAACG TAATAAAAAA ATTCTATACA
GATGATGAGT TAGAGGCTGT TTTAGTAGCT AAATCAGATG TACTTGTTGA
TGCTTTAGCA GCAGGTCCAT TGGCTGCGAA CTTAAAATCT CCAATACTTA
TAACACCAAA GACGTATGTA TCTGCATACC ATAAAGATAA TTTAGAAGCT

AAATCAGCTA ATAAGGTATA CAAAATAGGA GGAGGATTGA CTTCTAAGGT
AATGAGCTCT ATAGCATCAT CATTATCTAA ACACAATACG ACTCCAACAG
AACCAGGAAA TAGTGGGGGC AAGACAGTTA TGATTGACCC AGGGCATGGT
GGTTCAGCAC CTGGAAATTC ATCTGGAGGA ATGATTGAAA AAGATTACAA
TTTAAATACT TCACTTGCAA CAACTGAATA TTTACGTTCA AAGGGATTCA
ATGTAATAAT GACAAGAGAC ACAGATAAGA CTTTATCTCT TGGAAATAGA
ACTGCTCTAT CTAATTCATT GAAACCAGAT TTATTTACAA GTATACATTA
TAATGGCTCA ACTAATAAAC AAGGTCATGG TGTAGAAGTA TTTTATAAGC
TTAAAGATAA AAATGGAGGG ACTACTAAAA CTGTAGCTAC CAATATATTA
AATAGAATTT TAGAGAAATT TAAACTTACA AATAGAGGTA TAAAAACAAG
AGTACTTCCT AGTGATTCTA CAAAAGATTA TTTATACGTT TTAAGAAGTA
ATGATATGCC AGCTGTACTT GTAGAATGTG CATTTTTGGA TAATGAAAAT
GATATGAGTT TAATAAACTC ATCTGCAAAA GTAAAAGAAA TGGGTACACA
AATAGGTAAA GGAATAGAAG ATTCATTAAA A (SEQ ID NO:43). The translated amino acid sequence of CD1036 fragment in plasmid pCD1036-2 is:

MVSKEINMRRNTKLLTTGILSMAIVAPTMAFATESNAMENNADLNINLEKKSIVLGSK
SKVSVKFKEKPDADSITLKYKCYDMPLNTTLNYNQSTGAYEGTINYNQDPEYLNVWEL
QGITINSKNNHKTLNRQDLEKLGLNLKDYNVTQECIIEDITSRKDVNKYLRKTSSPIT
ELTGSDRYETAVKISKEGWKNGSDKVVIINGDVSIDGIISTPLATTYNAPILLVEKNN
VPNSVKSELKRLNPKDIIIIGDENAISKTTANQIKSTVNASQTRLNGSNRYETSLLIA
KEIDKNHDVEKVYITNANGGEVDALTIAAKAGQDKQPIILTDKDSITDNTYKWLKSED
LQNAYFIGGPQMISTNVINKVNGITKDSVTNNRVYGADRHETNANVIKKFYTDDELEA
VLVAKSDVLVDALAAGPLAANLKSPILITPKTYVSAYHKDNLEAKSANKVYKIGGGLT
SKVMSSIASSLSKHNTTPTEPGNSGGKTVMIDPGHGGSAPGNSSGGMIEKDYNLNTSL
ATTEYLRSKGFNVIMTRDTDKTLSLGNRTALSNSLKPDLFTSIHYNGSTNKQGHGVEV
FYKLKDKNGGTTKTVATNILNRILEKFKLTNRGIKTRVLPSDSTKDYLYVLRSNDMPA
VLVECAFLDNENDMSLINSSAKVKEMGTQIGKGIEDSLK (SEQ ID NO:49).

The nucleotide sequence of *C. difficile* ATCC 9689 CD1036 fragment (294 to 393 amino acid residues) in plasmid pCD1036-Fr1-1 is:

ATGGATAAAA ATCATGATGT GGAAAAAGTA TACATAACAA ATGCTAATGG
CGGAGAAGTG GATGCACTTA CTATAGCAGC AAAAGCAGGT CAAGACAAGC
AACCAATTAT ATTAACTGAT AAAGATAGTA TTACAGACAA TACATATAAA
TGGTTAAAGA GTGAGGATTT ACAAAATGCT TATTTTATAG GTGGTCCTCA

AATGATATCA ACAAATGTTA TAAATAAGGT AAATGGAATA ACTAAAGATA GTGTTACTAA TAATAGAGTA TACGGAGCAG ATAGACACGA AACAAATGCA AAC (SEQ ID NO:44).

The translated amino acid sequence 9689 CD1036 fragment (294 to 393 amino acid residues) in plasmid pCD1036-Fr1-1 is:

MDKNHDVEKVYITNANGGEVDALTIAAKAGQDKQPIILTDKDSITDNTYKWLKSEDLQ NAYFIGGPQMISTNVINKVNGITKDSVTNNRVYGADRHETNAN (SEQ ID NO:50).

Example 19

Expression of *C. difficile* ATCC 9689 amidase clones

The entire coding sequence of a putative N-acetylmuramoyl-L-alanine amidase cell surface protein from the recombinant plasmid pCD1036-2, as obtained in Example 18, and the expression vector pET21-d(+) (Novagen) were cut with the restriction enzymes NcoI and XhoI (New England Labs, Ipswich, MA) and cloned into pET21-d(+), using the procedures described in Example 17. Similarly, the fragment corresponding to amino acid residues 294 to 393 of the putative N-acetylmuramoyl-L-alanine amidase cell surface protein of *C. difficile* 630 (SEQ ID NO:6) from the recombinant plasmid pCD1036-Fr-1 was cut with the restriction enzymes NcoI and XhoI and cloned in to pET21-d(+), following the procedures described in Example 17. The recombinant clones were picked and analyzed as described in example 17. The clones having the inserts were selected and tested for expression of proteins according to manufacturer's instruction.

The recombinant clones were transformed into competent cells of BLR(DE3) and plated on LB Agar with ampicillin (50 μg/ml). Several colonies were picked and analyzed for protein expression as described in Example 17. The induced cells showed better expression of recombinant protein of expected molecular weight at 15° C than at 37° C.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in

GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

Sequence Listing Free Text

SEQ ID NO:1	hypothetical protein CD1021 of *C. difficile* strain 630
SEQ ID NO:2	residues 505-604 of hypothetical protein CD1021
SEQ ID NO:3	residues 203 to 217 hypothetical protein CD1021
SEQ ID NO:4	residues 333 to 347 hypothetical protein CD1021
SEQ ID NO:5	putative N-acetylmuramoyl-L-alanine amidase of *C. difficile* 630
SEQ ID NO:6	residues 294-393 of putative N-acetylmuramoyl-L-alanine amidase
SEQ ID NO:7	residues 582-596 of putative N-acetylmuramoyl-L-alanine amidase
SEQ ID NO:8	residues 64-78 of putative N-acetylmuramoyl-L-alanine amidase
SEQ ID NO:9	residues 30-120 of hypothetical protein CD1021
SEQ ID NO:10	residues 194-293 of hypothetical protein CD1021
SEQ ID NO:11-12	Amino acid sequence encoded in genome of *C. difficile* QCD-32g58
SEQ ID NO:13-14	Genomic sequence from *C. difficile* QCD-32g58
SEQ ID NO:15	Genomic sequence from *C. difficile* QCD-66c26
SEQ ID NO:16-17	Amino acid sequence encoded in genome of *C. difficile* strain 630
SEQ ID NO:18-20	Amino acid sequence encoded in genome of *C. difficile* QCD-32g58
SEQ ID NO:21	Translated amino acid sequence of *C. difficile* QCD-32g58
SEQ ID NO:22	Translated amino acid sequence of *C. difficile* QCD-32g58 genomic sequence
SEQ ID NO:23	Translated amino acid sequence of *C. difficile* QCD-66c26
SEQ ID NO:24-37	Synthetic oligonucleotide primers
SEQ ID NO:38	Consensus sequence
SEQ ID NO:39-44	Genomic sequence from *C. difficile* ATCC 9689

SEQ ID NO:45-50 Translated amino acid sequence of *C. difficile* ATCC 9689

What is claimed is:

1. An isolated antibody that binds to a *Clostridium difficile* spore.

2. The isolated antibody of claim 1 wherein the spore is an ungerminated spore.

3. The isolated antibody of claim 1 wherein the spore is a germinated spore.

4. The antibody of any one of claims 1 to 3, wherein the antibody does not bind to *Clostridium difficile* vegetative cells.

5. The isolated antibody of any one of claims 1 to 3, wherein the isolated antibody does not bind to *C. difficile* toxin.

6. An isolated antibody that binds to hypothetical protein CD1021 of *Clostridium difficile* strain 630 having SEQ ID NO: 1, or a fragment of hypothetical protein CD1021.

7. The isolated antibody of claim 6, wherein the isolated antibody binds a fragment of hypothetical protein CD1021 comprising amino acid residues 505 to 604.

8. An isolated antibody that binds to the amino acid sequence SEQ ID NO:2.

9. The isolated antibody of claim 6, wherein the isolated antibody binds a fragment of hypothetical protein CD1021 comprising amino acid residues 30 to 120.

10. An isolated antibody that binds to the amino acid sequence SEQ ID NO:9.

11. The isolated antibody of claim 6, wherein the isolated antibody binds a fragment of hypothetical protein CD1021 comprising amino acid residues 194 to 293.

12. An isolated antibody that binds to the amino acid sequence SEQ ID NO:10.

13. The isolated antibody of claim 6, wherein the isolated antibody binds to a fragment of hypothetical protein CD1021 comprising amino acid residues 203 to 217

14. The isolated antibody of claim 6, wherein the isolated antibody binds to a fragment of hypothetical protein CD1021 comprising amino acid residues 333 to 347.

15. An isolated antibody that binds to the amino acid sequence EGSSLQYKGDDPESY (SEQ ID NO:3).

16. An isolated antibody that binds to the amino acid sequence LKNETYKTKYHKYLE (SEQ ID NO:4).

17. An isolated antibody that binds to putative N-acetylmuramoyl-L-alanine amidase protein of *Clostridium difficile* strain 630 having SEQ ID NO: 5 or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

18. The isolated antibody of claim 17, wherein the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein comprising amino acid residues 294 to 393.

19. An isolated antibody that binds to the amino acid sequence SEQ ID NO:6.

20. The isolated antibody of claim 17, wherein the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein comprising amino acid residues 582 to 596.

21. The isolated antibody of claim 17, wherein the isolated antibody binds to a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein comprising amino acid residues 64 to 78.

22. An isolated antibody that binds to the amino acid sequence YKLKDKNGGTTKTVA (SEQ ID NO:7).

23. An isolated antibody that binds to the amino acid sequence KFKEKPDADSIKLKY (SEQ ID NO:8).

24. The isolated antibody of any one of claims 1 to 23 wherein the isolated antibody is a polyclonal antibody.

25. The isolated antibody of any one of claims 1 to 23 wherein the isolated antibody is a monoclonal antibody.

26. A monoclonal antibody, or antigen binding fragment thereof, wherein the monoclonal antibody or antigen binding fragment inhibits the binding of the monoclonal antibody of claim 25 to its antigen target.

27. The isolated antibody of any one of claims 1 to 26, wherein the isolated antibody does not bind to *Bacillus subtilis* spores or *Clostridium sporogenes* spores,

28. An antigen binding fragment of the isolated antibody of any one of claims 1 to 27.

29. The isolated antibody of any one of claims 1 to 27 or the antigen binding fragment of claim 28, wherein the isolated antibody or antigen binding fragment is labeled.

30. A composition comprising one or more of the isolated antibodies of claims 1 to 29.

31. A kit comprising one or more of the isolated antibodies of claims 1 to 29.

32. A hybridoma cell line or transformed B cell line that produces the monoclonal antibody of claim 25 or claim 26.

33. An isolated polynucleotide sequence comprising the nucleic acid sequence coding for the heavy chain, the light chain, the heavy chain variable region, the light chain variable region, or one or more complementarity determining regions of the monoclonal antibody of claim 25 or claim 26.

34. An expression vector comprising the isolated polynucleotide sequence of claim 33.

35. A host cell comprising the expression vector of claim 34.

36. A method of preparing an anti-*Clostridium difficile* antibody, the method comprising immunizing a host organism with a polypeptide comprising at least a portion of a protein encoded by the *C. difficile* genome in an amount effective to generate an antibody response to the polypeptide.

37. The method of claim 36 wherein the polypeptide comprising at least a portion of a protein encoded by the *C. difficile* genome is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

38. A method of preparing an anti-*Clostridium difficile* antibody, the method comprising

expressing a nucleic acid sequence encoding at least a portion of a protein encoded by the *C. difficile* genome in an immunocompetent host organism.

39. The method of claim 38 wherein the nucleic acid sequence encoding at least a portion of a protein encoded by the *C. difficile* genome encodes an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

40. The method of any one of claims 36 to 39 further comprising purifying the antibody preparation.

41. A composition comprising at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *Clostridium difficile* spore.

42. The composition of claim 41, wherein at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021.

43. The composition of claim 41, wherein at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

44. The composition of claim 41, wherein a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and wherein a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

45. The composition of claim 42 or claim 43, wherein the polypeptide fragment of hypothetical protein CD1021 is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

46. The composition of claim 43 or claim 45, wherein the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

47. A kit comprising at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *Clostridium difficile* spore.

48. The kit of claim 47, wherein at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021.

49. The kit of claim 47, wherein at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

50. The kit of claim 47, wherein a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and wherein a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

51. The kit of claim 48 or claim 50, wherein the polypeptide fragment of hypothetical protein CD1021 is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

52. The kit of claim 49 or claim 50, wherein the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

53. A method of detecting the presence of a *Clostridium difficile* spore in a sample, the method comprising contacting the sample with an isolated antibody of any one of claims 1 to 29.

54. A method of detecting the presence of a *Clostridium difficile* spore in a sample, the method comprising contacting the sample with at least two isolated antibodies or antigen-binding fragments thereof, wherein each isolated antibody binds to a distinct antigenic epitope of the *C. difficile* spore.

55. A method of detecting the presence of a *Clostridium difficile* spore in a sample, the method comprising:

contacting the sample with a first isolated antibody or antigen-binding fragment thereof, wherein the first isolated antibody binds to a first antigenic epitope of the *C. difficile* spore; and

contacting the sample with a second isolated antibody or antigen-binding fragment thereof, wherein the second isolated antibody binds to a second antigenic epitope of the *C. difficile* spore.

56. The method of claim 54 or 55, wherein at least one isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021.

57. The method of claim 54 or 55, wherein at least one isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

58. The method of claim 54 or 55, wherein a first isolated antibody or antigen-binding fragment thereof binds to hypothetical protein CD1021 of *C. difficile* strain 630 having SEQ ID NO: 1, or a polypeptide fragment of hypothetical protein CD1021, and wherein a second isolated antibody or antigen-binding fragment thereof binds to putative N-acetylmuramoyl-L-alanine amidase protein of *C. difficile* strain 630 having SEQ ID NO: 5, or a fragment of the putative N-acetylmuramoyl-L-alanine amidase protein.

59. The method of claim 56 or 58, wherein the polypeptide fragment of hypothetical protein CD1021 is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:10, and fragments thereof.

60. The method of claim 57 or 58, wherein the polypeptide fragment of the putative N-acetylmuramoyl-L-alanine amidase protein is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and fragments thereof.

```
SEQ ID NO.:
1          1 MKDKKFTLLISIMIVFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMMENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLISVANSDSDRYSYKINFDKYNT
23         1 MKDKKFTLLISIMIIFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMMENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLTSVANSDSDRYSYKINFDKYNT
21         1 .KIKKFTLLISIMIIFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMMENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLTSVANSDSDRYSYKINFDKYNT
11         1 ..............MIIFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMMENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLTSVANSDSDRYSYKINFDKYNT
12         1 ..................................................................................................................................
22         1 ..................................................................................................................................

Consensus  1 XXXXXXXXXXXXXXMIXFLCAVVGVYSTSSNKSVDLYSDVYIEKYFNRDKVMEVNIEIDESDLKDMMENAIKEEFKVAKVTVDGDTYGNVGIRTKGNSSLXSVANSDSDRYSYKINFDKYNT

1          121 SQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHGLYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKS
23         121 SQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHGLYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKS
21         120 SQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHGIYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKS
11         109 SQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHGLYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKS
12           1 ..................................................................................................................................
22           1 ..................................................................................................................................
Consensus 107 SQSMEGLTQLNLNNCYSDPSYMREFLTYSICEEMGLATPEFAYAKVSINGEYHGLYLAVEGLKESYLENNFGNVTGDLYKSDEGSSLQYKGDDPESYSNLIVESDKKTADWSKITKLLKS

1          241 LDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLKNETYKTKYHKYLEEIVTKYLDSDYLE
23         241 LDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLKNETHKTKYHKYLEEIVTKYLDSDYLE
21         240 LDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLK..............................
11         229 LDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLKK.............................
12           1 ..................................................................................................................................
22           1 ..................................................................................................................................
Consensus 227 LDTGEDIEKYLDVDSVLKNIAINTALLNLDSYQGSFAHNYYLYEQDGVFSMLPWDFNMSFGGFSGFGGGSQSIAIDEPTTGNLEDRPLISSLLKXXXXXXXXXXXXXXXXXXXXXXXXXXXX
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Ⓐ

Fig. 1a

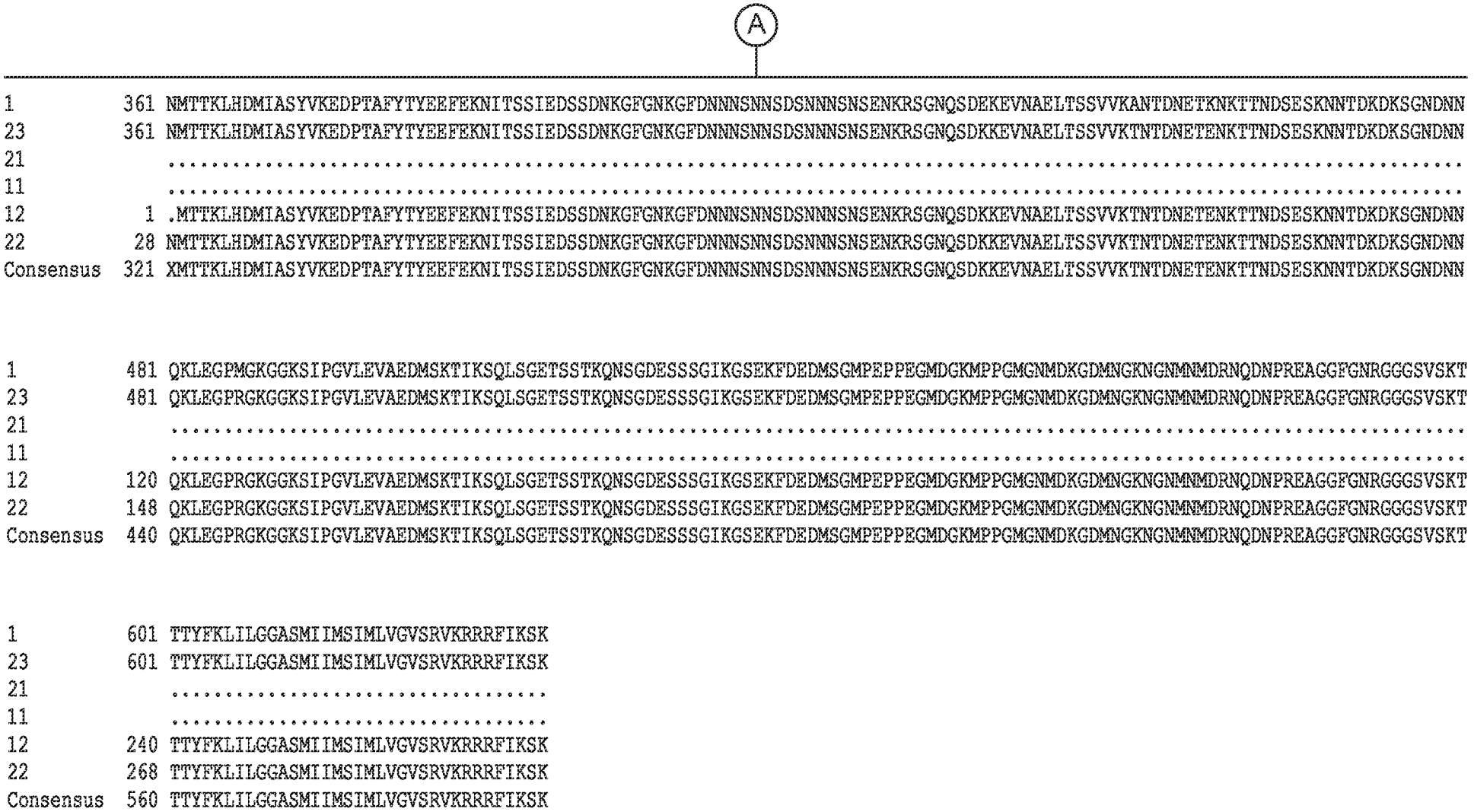
A
1 361 NMTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGNKGFDNNNSNNSDSNNNSNSENKRSGNQSDEKEVNAELTSSVVKANTDNETKNKTTNDSESKNNTDKDKSGNDNN
23 361 NMTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGNKGFDNNNSNNSDSNNNSNSENKRSGNQSDKKEVNAELTSSVVKTNTDNETENKTTNDSESKNNTDKDKSGNDNN
21 .....
11 .....
12 1 .MTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGNKGFDNNNSNNSDSNNNSNSENKRSGNQSDKKEVNAELTSSVVKTNTDNETENKTTNDSESKNNTDKDKSGNDNN
22 28 NMTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGNKGFDNNNSNNSDSNNNSNSENKRSGNQSDKKEVNAELTSSVVKTNTDNETENKTTNDSESKNNTDKDKSGNDNN
Consensus 321 XMTTKLHDMIASYVKEDPTAFYTYEEFEKNITSSIEDSSDNKGFGNKGFDNNNSNNSDSNNNSNSENKRSGNQSDKKEVNAELTSSVVKTNTDNETENKTTNDSESKNNTDKDKSGNDNN
1 481 QKLEGPMGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKT
23 481 QKLEGPRGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKT
21 .....
11 .....
12 120 QKLEGPRGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKT
22 148 QKLEGPRGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKT
Consensus 440 QKLEGPRGKGGKSIPGVLEVAEDMSKTIKSQLSGETSSTKQNSGDESSSGIKGSEKFDEDMSGMPEPPEGMDGKMPPGMGNMDKGDMNGKNGNMNMDRNQDNPREAGGFGNRGGGSVSKT
1 601 TTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK
23 601 TTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK
21 .....
11 .....
12 240 TTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK
22 268 TTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK
Consensus 560 TTYFKLILGGASMIIMSIMLVGVSRVKRRRFIKSK

Fig. 1b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/35050

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/40; G01N 33/554; C07H 21/04 (2009.04)
USPC - 424/167.1
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) - A61K 39/40; G01N 33/554; C07H 21/04 (2009.04)
USPC - 424/167.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/7.32; 435/69.1; 435/252.3; 435/340; 536/23.53

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB); Medline, Google
Search terms: Clostridium difficile, spore, C-difficile, ungerminated, germinated, vegetative, toxin, monoclonal, immunizing, polypeptide, expressing, nucleic acid, immunocompetent, host, purify, antigenic epitope, kit, distinct, antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO/1999/002188 A1 (SONGER, et al.) 21 January 1999 (21.01.1999), pg 4, ln 21-23; pg 4, ln 27-29; pg 5, ln 2-7; pg 5, ln 9-15; pg 8, ln 7-9; pg 22, ln 21-22	1-5, 41, 47 ---------- 54, 55
X	US 2005/0220783 A1 (LEE) 06 October 2005 (06.10.2005), para [0033], [0034], [0042], [0049]	36, 38
Y	US 2004/0033546 A1 (WANG) 19 February 2004 (19.02.2004), para [0046]-[0049], [0147], [0365], Table 3	54, 55

☐ 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 May 2009 (29.05.2009)	17 JUN 2009
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. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/35050

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.: 6-23, 37, 39, 40, 42-46, 48-52, 56-60
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:

Claims 6-23, 37, 39, 40, 42-46, 48-52 and 56-60 are unsearchable. The applicant failed to comply with the ISA/225 mailed on 16 March 2009. The CRF submitted on 07 April 2009 contained errors and could not be entered. Accordingly, the USPTO cannot supply a search for the sequences listed in this application.

3. ☒ Claims Nos.: 24-35, 53
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.