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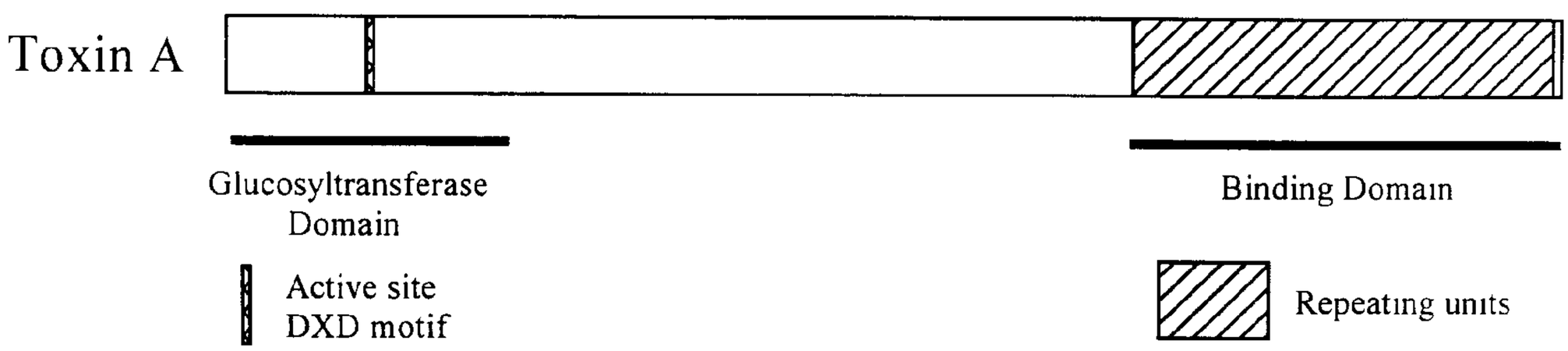
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(54) Title: RECOMBINANT CLOSTRIDIUM TOXIN A PROTEIN CARRIER FOR POLYSACCHARIDE CONJUGATE VACCINES



(57) Abrégé/Abstract:
The present invention provides for immunogenic compositions and their methods of use as vaccines and their method of preparation. These immunogenic compositions comprise a recombinant protein of toxin A of Clostridium difficile conjugated to a polysaccharide of a microbial pathogen. The immunogenic compositions may include only a truncated portion of toxin A, particularly the repeating units (rARU), that is conjugated to a microbial pathogen polysaccharide. Such compositions are effective in eliciting T-cell dependent and antibody responses. These compositions are therefore effective as vaccines for humans, particularly children, and animals in affording protection against one or more microbial pathogens.

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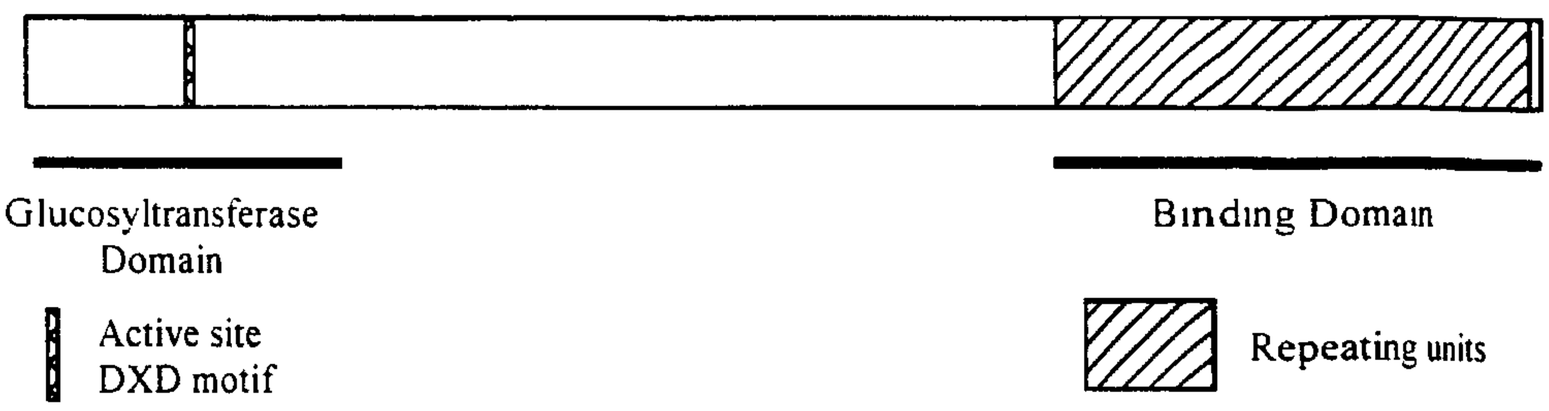
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(54) Title: RECOMBINANT CLOSTRIDIUM TOXIN A PROTEIN CARRIER FOR POLYSACCHARIDE CONJUGATE VACCINES



(57) Abstract: The present invention provides for immunogenic compositions and their methods of use as vaccines and their method of preparation. These immunogenic compositions comprise a recombinant protein of toxin A of *Clostridium difficile* conjugated to a polysaccharide of a microbial pathogen. The immunogenic compositions may include only a truncated portion of toxin A, particularly the repeating units (*r*ARU), that is conjugated to a microbial pathogen polysaccharide. Such compositions are effective in eliciting T-cell dependent and antibody responses. These compositions are therefore effective as vaccines for humans, particularly children, and animals in affording protection against one or more microbial pathogens.

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RECOMBINANT TOXIN A PROTEIN CARRIER FOR POLYSACCHARIDE CONJUGATE VACCINES

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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TECHNICAL FIELD OF THE INVENTION

The present invention relates to the field of medical immunology and further to pharmaceutical compositions, methods of making and methods of use of vaccines. More specifically this invention relates to a recombinant protein derived from a gene encoding *Clostridium difficile* toxin A, or closely related toxin B, as a carrier protein for enhancing the immunogenicity of a polysaccharide antigen.

BACKGROUND OF THE INVENTION

The development of effective vaccines has resulted in major advances for the prevention of many infectious diseases. Smallpox, for example, has been eliminated and the mention of polio, which has almost been completely eliminated, does not bring to the minds of younger generations the picture of crippling paralysis as it did several decades ago. The incidence of diphtheria, tetanus, measles, and whooping cough in many industrialized countries has been reduced significantly. Despite these advances, infectious diseases still remain the major cause of morbidity and mortality to the majority of persons around the world.

It is important that medical research continues to develop vaccines that are effective, inexpensive to produce and administer, and that exhibit minimal adverse side effects. Vaccination against pathogens is our first line of defense and represents a beneficial and cost-effective means of combating many infectious diseases. Therefore, it is imperative that collaborations such as the present one continue to develop new approaches for vaccines as well as improve those that we currently use.

Clostridium difficile, a Gram-positive anaerobic spore-forming bacillus, has been shown to be the etiologic agent of several forms of bacterial induced diarrhea. As part of a complex flora of the human intestinal tract, *C. difficile* has been shown to emerge as one of the causes of enteric microbial induced diarrhea following antibiotic therapy, which weakens or destroys many of the normal competitive enteric flora. Strains of *C. difficile* have been observed to cause only 25% of antibiotic-associated diarrheas, but have been found to be the causative agent of almost all cases of pseudomembranous colitis ("PMC"), some cases of which have been fatal (Lyerly, D.M. and T.D. Wilkins, in *Infections of the Gastrointestinal Tract*, Chapter 58, pages 867-891, (Raven Press, Ltd, New York 1995)). Additionally, *C. difficile* is frequently identified as a causative agent of nosocomial infectious diarrheas, particularly in older or immuno-compromised patients (U.S. Pat. No. 4,863,852 (Wilkins *et al.*) (1989)).

A significant component of the pathogenic repertoire of *C. difficile* is found in the two enteric toxins A and B produced by most strains (U.S. Pat. No. 5,098,826 (Wilkins *et al.*) (1992)). Toxin A is primarily an enterotoxin with minimal cytotoxic activity. While toxin B is a potent cytotoxin, the extensive damage to the intestinal mucosa is attributable to the action of toxin A, however, there are reports that toxins A and B may act synergistically in the intestine.

The genetic sequences encoding both toxigenic proteins A and B, the largest known bacterial toxins, with molecular weights of 308,000 and 269,000, respectively, have been elucidated (Moncrief *et al., Infect. Immun.* 65:1105-1108 (1997); Barroso *et al., Nucl. Acids Res.* 18:4004 (1990); Dove *et al. Infect. Immun.* 58:480-488 (1990)). Because of the degree of similarity when conserved substitutions are considered, these toxins are thought to have arisen from gene duplication. The proteins share a number of similar structural features with one another. For example, both proteins possess a putative nucleotide binding site, a central hydrophobic region, four conserved cysteines and a long series of repeating units at their carboxyl ends. The repeating units of toxin A, particularly, are immunodominant and are responsible for binding to type 2 core carbohydrate antigens on the surface of the intestinal epithelium (Krivan *et al., Infect. Immun.* 53:573-581 (1986); Tucker, K. and T.D. Wilkins, *Infect. Immun.* 59:73-78 (1991)).

The toxins share a similar molecular mechanism of action involving the covalent modification of Rho proteins. Rho proteins are small molecular weight effector proteins that have a number of cellular functions including maintaining the organization of the cytoskeleton. The covalent modification of Rho proteins is due to glucosyltransferase activity of the toxins. A glucose moiety is added to Rho using UDP-glucose as a cosubstrate (Just *et al. Nature* 375:500-503 (1995); Just *et al. J. Biol. Chem* 270:13932-13939 (1995)). The glucosyltransferase activity has been localized to approximately the initial 25% of the amino acid sequence of each of these toxins (Hofmann *et al. J. Biol. Chem.* 272:11074-11078 (1997); Faust and Song, *Biochem. Biophys. Res. Commun.* 251:100-105 (1998)) leaving a large portion of the toxins, including the repeating units, that do not participate in the enzymatic activity responsible for cytotoxicity.

The immunogenicity of the surface polysaccharides of bacterial pathogens is improved when these antigens are bound covalently to a carrier protein (conjugate). Conjugate vaccines against *Haemophillus influenzae* type b have virtually eliminated the disease in developed countries that routinely vaccinate children (Robbins, J.B., and R. Schneerson, *J. Infect. Dis.* 161:821-832 (1990);Robbins *et al., JAMA* 276:1181-1185 (1996)). This approach to improving the immunogenicity of polysaccharide antigens is based on experiments defining the effect of attaching a hapten (small molecule) or an antigen that is poorly immunogenic by itself to a carrier protein (Avery *et al., J. Exp. Med.* 50:521-533 (1929); Goebel, W.F., *J. Exp. Med.* 69:353-364 (1939); Buchanan-Davidson *et al., J. Immunol.* 83:543-555 (1959); Fuchs, *et al., J. Biol. Chem.* 240:3558-3567 (1965)). Conjugates containing polysaccharides from a number of different encapsulated pathogenic microorganisms have been tested in animals and humans and shown to elicit polysaccharide antibodies (Chu *et al., Infect. Immun.* 59:4450-4458 (1991); Devi *et al., Infect. Immun.* 59:732-736 (1991); Devi *et al., Infect. Immun.* 59:3700-3707 (1990); Fattom *et al., Infect. Immun.* 60:584-589 (1992); Fattom *et al., Infect. Immun.* 61:1023-1-32 (1993); Konadu *et al., Infect. Immun.* 62:5048-5054 (1994); Kayhty *et al. J. Infect. Dis.* 172:1273-1278 (1995); Szu *et al., Infect. Immun.* 54:448-453 (1986); Szu *et al., Infect. Immun.* 59:4555-4561(1991); Szu *et al., Infect. Immun.* 57:3823-3827 (1989)). Antibodies to surface polysaccharides induced by vaccination with conjugates may

confer protection against the encapsulated microorganism by inactivating the innoculum (Robbins *et al. J. Infect. Dis*. 171:1387-1398 (1995)).

Most carriers for conjugate vaccines have been medically useful proteins, namely, inactivated toxins of: tetanus, diphtheria, pertussis and *Pseudomonas aeruginosa* (Anderson *et al. J. Clin. Invest*. 76:52-59 (1985); Cohen *et al. Lancet* 349:155-159; Dagan *et al. Infect. Immun*. 66:2093-2098 (1998); Devi *et al. Proc. Natl. Acad. Sci USA* 88:7175-7179 (1991); Pavliakova *et al. Infect. Immun*. 67:5526-5529 (1999); Schneerson *et al. Infect. Immun*. 60:3528-3532 (1992)). Conjugate vaccines, therefore, may confer protection against pathogens whose protective antigens are the carrier proteins, including those that cause toxin-mediated diseases. In cases where tetanus toxin has been used, toxin-neutralizing antibody responses have been observed (Claesson *et al. J. Pediatr*. 112:695-702 (1988); Lagergard *et al. Infect. Immun*. 58:687-694 (1990); Schneerson *et al. Infect Immun*. 52:519-528 (1986)). Further, tetanus toxin (molecular weight 150,000) is twice the size of either diphtheria toxin or exotoxin A from *Pseudomonas aeruginosa* and results in a higher level of antibody produced against the polysaccharide antigenic component (Robbins, J.B. and R. Schneerson, *J. Infect. Dis.*, 161:821-832 (1990)).

Proteins derived from toxin A and B of *C. difficile* may be candidates for a carrier protein that may be useful for conjugate vaccines against nosocomial infections by serving as effective carriers for polysaccharides. Examples of encapsulated nosocomial pathogens that could likely be protected against by *r*ARU conjugate vaccines include: *Staphylococcus aureus*; coagulase-negative *Staphylococcus*; *Enterococcus* species; *Enterobacter* species; *Candida* species; group B *Streptococcus; Escherichia coli;* and *Pseudomonas* species.

Nosocomial infections due to *S. aureus* and *C. difficile* represent a major health care problem in the United States. This is particularly true in light of the emerging threat posed by antibiotic resistant pathogens such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant Enterococci (VRE) (Thornsberry C. *West J. Med*. 164:28-32 (1996) that may transfer resistance to MRSA. The incidence of *S. aureus* infections continues to rise and it is currently the most common cause of death from nosocomial infections (Weinstein, RA Eme1998). Its prevalence, in part, is due to the wide range of infections it causes and its extensive repertoire of virulence

factors (Archer, GL *Clin. Infect. Dis.* 26:1179-1181 (1998)). Further, strains of *S. aureus* are commonly carried in the nasal passages and on the skin making it exceedingly difficult to control the spread of this organism. In addition to causing hospital-acquired infections, *S. auerus* is becoming more commonly recognized as a community-acquired infection (Kayaba *et al. Surg Today* 27:217-219 (1997); Moreno *et al. Clin Infect. Dis*. 212:1308-1312(1995)). Strains of *S. aureus* that are increasingly virulent and resistant to antibiotic therapy continue to emerge. Recently strains with intermediate resistance to vancomycin have been identified in the U.S. and other developed nations (Tenover *et al. J. Hosp Infect* 43 Suppl:S3-7 (1999); Woodford *et al. J. Antimicrob Chemother*. 45:258-259 (2000)). This is an alarming development, since vancomycin resistant strains of *S. aureus* that are also multiply resistant to other antibiotics would be exceedingly difficult to treat without the development of novel therapies.

Serotypes 5 and 8 cause about 85% of *S. aureus* infections and experimental evidence suggests that antibodies to capsular polysaccharides of *S. aureus* may protect against disease (Fattom *et al. Infect. Immun*. 58:2367-2374 (1990); Fattom *et al. Infect. Immun.* 61:1023-1024 (1993)). Therefore, a conjugate vaccine against serotypes 5 and 8 may be broadly protective. Further, in the case of *H. influenzae* type b (Hib) conjugate vaccines, vaccination has decreased the carriage of *H. influenzae* in the nasal passages. This is thought to have contributed to the success of Hib conjugate vaccines through herd immunity (Robbins *et al .JAMA* 276:1181-1185 (1996)). A similar effect may be seen with an effective conjugate vaccine against *S. aureus*, which may be particularly important for eliminating hospital acquired infections by vaccinating health care workers as well as patients.

Conjugate vaccines are also considered to provide epitopes to polysaccharide antigens that may be recognized by T helper cells (Avery O.T. and W.F.Goebel *J. Experimental Med*. 50:533-550 (1929)). A strong antibody response appears to require an interaction of antigen-specific B cells with T helper cells. This event is thought to be essential in a humoral immune response that leads to production of large amounts of high avidity antibodies and the formation of immunological memory. In this event B cells act as antigen presenting cells (APCs). Unlike other APCs, however, B cells take up antigen in a specific manner by binding the antigen with

antibodies on the surface of the cell. These B cells are capable of differentiating into plasma cells that secrete antibody to the antigen. Also, a subpopulation of activated B cells differentiate into memory cells that are primed to recognize the antigen and become activated upon subsequent exposure. In both cases differentiation requires direct interaction with T helper cells. Upon uptake of the antigen, B cells process the antigen (protein) and present T cell epitopes on the surface in context with MHC class II. Antigen specific T helper cells then bind the T helper epitope/MHC class II complex and release helper cytokines leading to the differentiation of B cells into antibody secreting plasma cells or memory cells. The event also leads to differentiation of the specific T helper cells into memory cells. The immune system is therefore primed for an anamnestic response (booster effect) upon subsequent exposure to the antigen.

Polysaccharide antigens do not contain T cell epitopes. Polysaccharides, therefore, induce a T cell-independent response when presented without an attached protein. The T cell-independent response results in short lived antibody responses characterized by low affinity antibodies predominated by IgM. Conjugation of a protein to the polysaccharide provides T cell epitopes to the polysaccharide. This converts the T cell-independent response to a T cell-dependent response. Upon uptake of the conjugate by B cells specific for the polysaccharide the protein portion of the conjugate is processed and T cell epitopes are displayed on the surface of the B cell in context with MHC class II for interaction with T helper cells. Therefore, B cells that secrete antibody to the polysaccharide are expanded in a T cell-dependent manner.

*r*ARU is comprised of 31 contiguous repeating units and may contain multiple T cell epitopes (Dove *et al. Infect. Immun.* 58:480-488 (1990). The repeating units are defined as class I and class II repeats. *r*ARU may be uniquely suited for use in inducing T cell-dependent response to polysaccharides. The sequence of each unit is similar but not identical.

The toxin B repeating units have similar features to those of *r*ARU. Like *r*ARU, the recombinant toxin B repeating units (*r*BRU) are relatively large (~70 kDa) and are composed of contiguous repeats of similar amino acid sequences (Barroso *et al. Nucleic Acids Res.* 18:4004 (1990); Eichel-Streiber *et al. Gene* 96:107-113

(1992)). Less is known about this portion of toxin B than the binding domain of toxin A. Thomas et al (U.S. Pat. No. 5,919,463 (1999)) disclose *C. difficile* toxin A or toxin B or certain fragments thereof as mucosal adjuvants intranasally administered to stimulate an immune response to an antigen (e.g., Helicobacter pylori urease, ovalbumin (OVA), or keyhole limpet hemocyanin (KLH)).

Even were one to consider *r*ARU and *r*BRU as candidate carrier proteins for conjugate vaccines, the production of such proteins presents certain challenges. There are methods for the production of toxin A and antibodies elicited thereto (U.S. Pat. No. 4,530,833 (Wilkins *et al.*)(1985); U.S. Pat. No. 4,533,630 (Wilkins *et al.*)(1985); and U.S. Pat. No. 4,879,218 (Wilkins *et al.*)(1989)). There are significant difficulties in producing sufficient quantities of the *C. difficile* toxin A and toxin B proteins. These methods are generally cumbersome and expensive. However, the present invention provides for the construction and recombinant expression of a nontoxic truncated portions or fragments of *C. difficile* toxin A and toxin B in strains of *E. coli*. Such methods are more effective and commercially feasible for the production of sufficient quantities of an efficient carrier molecule for raising humoral immunogenicity to polysaccharide antigens.

Part of the difficulty that the present invention overcomes concerns the fact that large proteins are difficult to express at high levels in *E. coli*. Further, an unusually high content of AT in these clostridial gene sequences (i.e., AT-rich) makes them particularly difficult to express at high levels (Makoff *et al. Bio/Technology* 7:1043-1046 (1989)). It has been reported that expression difficulties are often encountered when large (i.e., greater than 100 kd) fragments are expressed in *E. coli*. A number of expression constructs containing smaller fragments of the toxin A gene have been constructed, to determine if small regions of the gene can be expressed to high levels without extensive protein degradation. In all cases, it was reported that higher levels of intact, full length fusion proteins were observed rather than the larger recombinant fragments (Kink et al., U.S. Pat. No. 5,736,139; see: Example 11(c)). It has been further reported that AT-rich clostridial genes contain rare codons that are thought to interfere with their high-level expression in *E. coli* (Makoff *et al. Nucleic Acids Research* 17:10191-10202). The present invention provides for methods to produce genes that are both large and AT-rich. For example, the toxin A repeating

units are approximately 98 kDa and the gene sequence has an AT content of approximately 70% that is far above the approximately 50% AT content of the *E. coli* geneome. The present invention provides for methods of expressing AT-rich genes (including very large ones) at high levels in *E. coli* without changing the rare codons or supplying rare tRNA.

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents. Further, all documents referred to throughout this application are incorporated in their entirety by reference herein. Specifically, the present application claims benefit of priority to U.S. provisional patent application serial number 60/186,201, which was filed on March 1, 2000, and U.S. provisional patent application serial number 60/128,686, which was filed on April 9, 1999, and which provisional patent applications are incorporated in their entirety by reference herein.

SUMMARY OF THE INVENTION

The present invention is drawn to an immunogenic composition that includes a recombinant protein component and a polysaccharide component. The gene encoding the protein component is isolated from a strain of *C. difficile*. The polysaccharide component is not a *C. difficile* polysaccharide and is isolated from a source other than *C. difficile*.

A preferred embodiment of this invention provides that the protein component is a toxin or a toxin fragment. A further preferred embodiment provides that the toxin is *C. difficile* toxin A. A more preferred embodiment of the present invention provides that the protein component comprise all the amino acid sequence of the *C. difficile* toxin A repeating units (*r*ARU) or fragment thereof. The immunogenic composition may further include a pharmaceutically acceptable carrier or other compositions in a formulation suitable for injection in a mammal.

Another preferred embodiment provides that the toxin is *C. difficile* toxin B. A further preferred embodiment provides that the protein is comprised of a portion of toxin B that includes the repeating units (*r*BRU) of the toxin or a fragment thereof.

Another embodiment of the present invention includes methods for producing an immunogenic composition by: constructing a genetic sequence encoding a recombinant protein component where the gene encoding the protein component is isolated from a strain of *C. difficile*; expressing the recombinant protein in a microbial host; recovering the recombinant protein component from a culture of the microbial host; conjugating the protein component to a polysaccharide component, where the polysaccharide component is isolated from a source other than *C. difficile*; and recovering the conjugated protein component and polysaccharide component. A preferred embodiment provides that the polysaccharide component is isolated from a pathogenic microorganism or is chemically synthesized. A still further preferred embodiment of this invention includes maintaining expression of the genetic sequence encoding the protein component in the microbial host throughout the growth of the host cell by constant and stable selective pressure.

A further preferred embodiment of this invention provides that the pathogenic microorganism is selected from the group consisting of: *Streptococcus pneumoniae*; *Neisseria meningitidis*; *Escherichia coli*; and *Shigella* species. An even further preferred embodiment is that the pathogenic microorganism consists of an encapsulated microbial pathogen that causes nosocomial infections including: *Staphylococcus aureus;* coagulase- negative *Staphylococcus* species; *Enterococcus* species; *Enerobacter* species; *Candida* species; *Escherichia coli*; and *Pseudomonas* species.

Another embodiment of this invention includes an expression vector and transformed microbial host cell, where the expression vector comprises the gene encoding the protein component. A preferred embodiment provides that the gene encoding the protein component is operably linked to one or more controllable genetic regulatory expression elements. An even further preferred embodiment provides that the gene encoding the protein component is fused to a second genetic sequence, the expression of which results in the production of a fusion protein. A still further preferred embodiment includes that the controllable genetic regulatory expression

elements comprise an inducible promoter sequence that is operatively positioned upstream of the gene encoding the protein component and the inducible promoter sequence is functional in the microbial host. An even further preferred embodiment of the present invention includes a selective phenotype encoded on the expression vector by an expressible genetic sequence, the expression of which in the microbial host results in stable growth of the microbial host and constant production of the protein component when the host is cultured under conditions for which the selective phenotype is necessary for growth of the microbial host. A still further preferred embodiment includes a selectable phenotype that confers drug resistance upon the microbial host, while an even further preferred embodiment provides that the drug resistance gene is a kanamycin resistance gene, the expression of which enables the microbial host to survive in the presence of kanamycin in the culture medium.

The methods and compositions of the present invention also provide for a level of expression of the recombinant protein in the microbial host at a level greater than about 10 mg/liter of the culture, more preferably greater than about 50 mg/liter and even more preferably at 100 mg/liter or greater. The molecular weight of the protein is greater than about 30 kDa, preferably greater than about 50 kDa and even more preferably greater than about 90 kDa. This invention also provides that the protein may be recovered by any number of methods known to those in the art for the isolation and recovery of proteins, but preferably the recovery is by ammonium sulfate precipitation followed by ion exchange chromatography.

The present invention further includes methods for preparing the immunogenic composition that provides that the protein is conjugated to the polysaccharide by one of a number of means known to those in the art, but preferably by first derivatizing the protein by succinylation and then conjugating the polysaccharide component to the protein through a reaction of the protein and polysaccharide component with 1, ethyl-3-(3-dimethylaminopropyl) carboiimide hydrochloride. Additionally the invention contemplates the activation of the polysaccharide component by the use of any of several reagents, but preferably cyanogen bromide. The polysaccharide may be further derivatized by adipic acid dihydrazide. Conjugates synthesized with *r*ARU may also be prepared by reductive

amination or any other methods known in the art (Gray *GR Methods Enzymol* 50:155-160 (1978); Pawlowski *et al. Vaccine* 17:1474-1483).

The present invention further includes methods of use of compositions of this invention for the treatment of mammalian subjects infected with a pathogenic microorganism. Similarly, this invention provides methods of use of compositions of the present invention to provide protection against infection of a mammalian subject by a pathogenic microorganism.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic of *Clostridium difficile* toxins A and B. The enzymatic activity responsible for the cytotoxicity of toxins A and B is contained in the N-terminal glucosylyltransferase domain (Just *et al. Nature* 375:500-503 (1995); Just *et al. J. Biol. Chem* 270:13932-13939 (1995)). A DXD motif common to glycosyltransferases is essential for enzymatic activity (Busch *et al. J. Biol. Chem* 273:19566-19572 (1998)). The enzymatic domain and middle region of the toxin are deleted from the toxin A gene fragment encoding *r*ARU (toxin A repeating units comprising the binding domain). The small open box at the end of toxin A represents a small stretch of hydrophobic amino acids.

Fig. 2 shows the nucleotide sequence (numbers 5690-8293, GenBank accession number M30307, Dove *et al.* 1993) of the toxin A gene region that encodes *r*ARU and the toxin A stop codon. The sequence encodes for the entire repeating units of toxin A from *C. difficile* strain VPI 10463 as defined by Dove *et al.* (Dove *et al.*, *Infect Immun.* 58:480-488 (1990)). In addition it encodes for 4 amino acids upstream of the beginning of the repeating units and a small stretch of hydrophobic amino acids at the end of toxin A. The Sau3A site (underlined) at the beginning of the sequence was used to subclone the gene fragment to an expression vector. The stop codon at the end of the sequence is italicized.

Fig. 3 shows the amino acid sequence (GenBank accession number M303307) of *r*ARU. The invention contemplates the use of any recombinant protein containing this amino acid sequence, any fragment therein, any fusion protein containing *r*ARU or a fragment therein, and any larger fragment from toxin A carrying all or part of *r*ARU, as a carrier for conjugate vaccine compositions.

Fig. 4 shows the expression vector pRSETB-ARU-Km^r used for expression of *r*ARU. A Sau3A/HindIII gene fragment of approximately 2.7 kb containing the entire nucleotide sequence encoding *r*ARU, stop codon, and a small region downstream of the toxin A stop codon, was subcloned to the vector pRSETB digested with BamHI and HindIII. In a subsequent step the kanamycin resistance gene was subcloned at the HindIII site located downstream of the *r*ARU gene fragment. The 1.2 kb fragment encoding the Km^r gene was derived from pUC4K (GenBank accession number X06404) by digestion with EcoRI and subcloned at the HindIII site after blunt ending of the vector and Km^r cassette with Klenow fragment. Expression vector pRSETB-ARU-Km^r was transformed into BL21(DE3) for expression of *r*ARU under control of the T7 promoter.

* HindIII/EcoRI sites were eliminated by blunt ending.

Fig. 5 shows an SDS-PAGE gel (15% acrylamide) of *r*ARU expression and purification steps. Lanes: 1) 4 µl of 10X BL21(DE3) *E. coli*/pRSETB-ARU-Km^r lysate 2) 4 µl of dialyzed 40% ammonium sulfate fraction at 10X relative to the original culture volume 3) 5 µl *r*ARU (0.88 mg/ml) purified by CL-6B Sepharose anion exchange chromatography.

Fig. 6 shows the chemical structure of polysaccharides conjugated to *r*ARU. Pneumococcal type 14 is a neutral high molecular weight branched copolymer (Lindberg *et al. Carbohydr. Res.* 58:177-186 (1977)), *Shigella flexneri* 2a O-specific polysaccharide is a comparatively lower molecular weight neutral branched copolymer (Carlin et al. *Eur. J. Biochem.* 139:189-194 (1984); Kenne *et al. Eur. J. Biochem*. 91:279-284 (1978)), and each subunit of *E. coli* K1, a linear high molecular weight homopolymer, is negatively charged (Bhattacharjee *et al. J. Biol. Chem.* 250:1926-1932 (1975)). Conjugation of each polysaccharide to *r*ARU resulted in high-level antibody responses. Thus, the use of *r*ARU as a carrier is likely to be applicable to all polysaccharides.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is drawn to an immunogenic composition that includes a recombinant protein component and a polysaccharide component. The gene encoding

the protein component is isolated from a strain of *C. difficile*. The polysaccharide component is not a *C. difficile* polysaccharide and is isolated from a source other than *C. difficile*. The polysaccharide is medically useful and is isolated from a pathogenic microorganism or synthesized. A preferred embodiment of this invention provides that the protein is a toxin or a toxin fragment. An even further preferred embodiment provides that the toxin is toxin A, with yet a further preferred embodiment being a portion of the toxin containing all of the amino acid sequence of the toxin A repeating units (*r*ARU) or fragment thereof. Another preferred embodiment is that the toxin is toxin B, with yet another preferred embodiment being a portion of the toxin containing all of the amino acid sequence of the repeating units (*r*BRU) or a fragment thereof. The immunogenic composition may further include a pharmaceutically acceptable carrier or other compositions in a formulation suitable for injection in a mammal.

These immunogenic compositions of the present invention elicit an immune response in a mammalian host, including humans and other animals. The immune response may be either a cellular dependent response or an antibody dependent response or both and further the response may provide immunological memory or a booster effect or both in the mammalian host. These immunogenic compositions are useful as vaccines and may provide a protective response by the mammalian subject or host to infection by a pathogenic microorganism.

The present invention further includes methods for producing an immunogenic composition by: constructing a genetic sequence encoding a recombinant protein, where the gene encoding the protein is isolated from a strain of *C. difficile*; expressing the recombinant protein in a microbial host; recovering the recombinant protein from a culture of the host; conjugating the protein to a polysaccharide component, wherein the polysaccharide component is isolated from a source other than *C. difficile*; and recovering the conjugated protein and polysaccharide component. The protein component may also consist of a fusion protein, whereby a portion of the said recombinant protein is genetically fused to another protein. Preferably the expression of the genetic sequence is regulated by an inducible promoter that is operatively positioned upstream of the sequence and is functional in the host. Even further, the said genetic sequence is maintained throughout the growth of the host by constant and

stable selective pressure. Maintenance of the expression vector may be conferred by incorporation in the expression vector of a genetic sequence that encodes a selective genotype, the expression of which in the microbial host cell results in a selective phenotype. Such selective genotypes, include a gene encoding resistance to antibiotics, such as kanamycin. The expression of this selective genotypic sequence on the expression vector in the presence of a selective agent or condition, such as the presence of kanamycin, results in stable maintenance of the vector throughout growth of the host. A selective genotype sequence could also include a gene complementing a conditional lethal mutation.

Other genetic sequences may be incorporated in the expression vector, such as other drug resistance genes or genes that complement lethal mutations.

Microbial hosts of this invention may include: Gram positive bacteria; Gram negative bacteria, preferably *E. coli*; yeasts; filamentous fungi; mammalian cells; insect cells; or plant cells.

The methods of the present invention also provide for a level of expression of the recombinant protein in the host at a level greater than about 10 mg/liter of the culture, more preferably greater than about 50 mg/liter and even more preferably at 100 mg/liter or greater than about 100 mg/liter. The molecular weight of the protein is greater than about 30 kDa, preferably greater than about 50 kDa and even more preferably greater than about 90 kDa. This invention also provides that the protein may be recovered by any number of methods known to those in the art for the isolation and recovery of proteins, but preferably the recovery is by ammonium sulfate precipitation followed by ion exchange chromatography.

The present invention further includes methods for preparing the immunogenic composition that provides that the protein is conjugated to the polysaccharide by one of a number of means known to those in the art, but preferably by first derivatizing the protein by succinylation and then conjugating the polysaccharide component to the protein through a reaction of the protein and polysaccharide component with 1, ethyl-3-(3-dimethylaminopropyl) carboiimide hydrochloride. Additionally the invention contemplates the activation of the polysaccharide component by the use of any of several reagents, but preferably

cyanogen bromide. The polysaccharide may be further derivatized by adipic acid dihydrazide.

A number of polysaccharides components may be selected and conjugated to the protein component of the present invention. The immunogenic compositions of the present invention may further comprise a polysaccharide, lipopolysaccharide, capsular polysaccharide or other polysaccharide component. Such polysaccharide component may be selected, for example, from a pathogenic microorganism selected from the group consisting of: *Streptococcus pneumoniae; Shigella* species; and *Escherichia coli.*

Such polysaccharide components may be more specifically selected, for example, from a serotype of *Streptococcus pneumoniae*, selected from the group consisting of serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 25, and 33F. Also, the polysaccharide component may be selected from any species of *Shigella*, including, for example, *S. flexneri* and may include any serotype of a *Shigella* species, including *S. flexneri*, serotype 2a. The polysaccharide may be specifically selected from a type of *E. coli,* for example *E. coli* K1.

The polysaccharide component may also be selected from any nosocomial pathogenic microorganism, from the group consisting of: *Staphylococcus aureus*; coagulase-negative *Staphylococcus* species; *Enterococcus* species; *Enterobacter* species; *Candida* species; group B *Streptococcus; Escherichia coli;* and *Pseudomonas* species.

Polysaccharide components may be more specifically selected, for example, from serotypes of *S. aureus*, including, for example, *S. aureus* serotype 5 or *S. aureus* serotype 8.

Also, high yields of recombinant protein may be dependent on the growth conditions, the rate of expression, and the length of time used to express the AT-rich gene. In general, AT-rich genes appear to be expressed at a higher level in *E. coli* during a post-exponential or slowed phase of growth. High-level production of the encoded protein requires moderate levels of expression over an extended period (e.g. 20-24 h) of post-exponential growth rather than the typical approach of high-level expression during exponential growth for shorter periods (e.g. 4-6 h). In this regard, it

is more efficient to maintain plasmids carrying the gene of interest by maintaining constant selective pressure for the gene or its expression vector during the extended period of growth. One aspect of the present invention is using an antibiotic that is not inactivated or degraded during growth of the expression host cell as is found with ampicillin. This embodiment involves the expression of genes encoding resistance to kanamycin as the selective phenotype for maintaining the expression vector which comprises such kanamycin resistance genetic sequences. Expression of large AT-rich clostridial genes in *E. coli* at levels (> 100 mg/liter) provided for by methods of the present invention was hitherto unknown.

Terms as used herein are based upon their art recognized meaning and should be clearly understood by the ordinary skilled artisan.

*r*ARU is a recombinant protein containing the repeating units of *Clostridium difficile* toxin A as defined by Dove *et al.* (Dove *et al. Infect. Immun.* 58:480-488 (1990)). The nucleotide sequence encoding *r*ARU and the amino acid sequence of *r*ARU are shown in Figs. 2 and 3, respectively. The *r*ARU expressed by pRSETB-ARU-Km^r contains the entire repeating units region of toxin A. The invention further contemplates the use of this recombinant protein, or any other protein containing the entire repeating units of toxin A or any fragment therein, whether expressed alone or as a fusion protein.

A fusion protein is a recombinant protein encoded by a gene or fragment of a gene, genetically fused to another gene or fragment of a gene.

An immunogenic composition is any composition of material that elicits an immune response in a mammalian host when the immunogenic composition is injected or otherwise introduced. The immune response may be humoral, cellular, or both.

A booster effect refers to an increased immune response to an immunogenic composition upon subsequent exposure of the mammalian host to the same immunogenic composition.

A humoral response results in the production of antibodies by the mammalian host upon exposure to the immunogenic composition.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way

of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLES

EXAMPLE 1

Construction of *r*ARU expression vector.

The vector pRSETB-ARU-Km^r used for expression and purification was constructed using standard techniques for cloning (Sambrook *et al., Molecular Cloning: A Laboratory Manual* (1989)). The nucleotide sequence of the toxin A gene fragment encoding *r*ARU was derived from the cloned toxin A gene (Dove *et al., Infect. Immun.* 58:480-488 (1990); Phelps *et al., Infect Immun.* 59:150-153 (1991)) and is shown in Fig. 2. The gene fragment encodes a protein 867 amino acids in length (Fig. 3) with a calculated molecular weight of 98 kDa. The gene fragment was subcloned to the expression vector pRSETB. A kanamycin resistance gene was subsequently subcloned to the vector. The resulting vector pRSETB-ARU-Km^r expresses *r*ARU. An additional 31 amino acids at the N-terminus of the recombinant protein are contributed by the expression vector pRSETB. The final calculated molecular weight of the recombinant protein is 102 kDa.

EXAMPLE 2

Expression and purification of *r*ARU.

Escherichia coli T7 expression host strain BL21(DE3) was transformed with pRSETB-ARU-Km^r as described (Sambrook *et al. Molecular Cloning: A Laboratory Manual* (1989)). One liter cultures were inoculated with 10 ml of overnight growth of *Escherichia coli* BL21(DE3) containing pRSETB-ARU-Km^r and grown at 37°C in Terrific broth (Sigma, St. Louis, MO) containing 25 μg/ml of kanamycin to an O.D. 600 of 1.8-2.0 and isopropyl B-D-thiogalactopyranoside (IPTG) was added to a final concentration of 40 μM. Cells were harvested after 22 h of induction, suspended in 0.1 liter of standard phosphate buffered saline, pH 7.4, containing 0.2 % casamino acids, and disrupted by sonication. Cellular debris was removed from the lysate by centrifugation. Lysates typically contained a titer (reciprocal of the highest dilution

with an A_{450} greater than 0.2) of 10^6 in the TOX-A test EIA (TechLab, Inc., Blacksburg, VA). Lysates were saturated with 40% ammonium sulfate, stirred at 4°C overnight and precipitating proteins were harvested by centrifugation. The ammonium sulfate fraction was suspended in 0.1 liters of 5 mM K_2PO_4, 0.1 M $NaCl_2$, pH 8.0 and dialyzed extensively against the same buffer at 4°C. Insoluble material was removed by centrifugation. The dialyzed solution was passed through a column containing Sepharose CL-6B chromatography media (50 ml media/100 ml solution). Fractions were collected and monitored for the presence of *r*ARU by EIA using the TOX-A test. Fractions containing EIA activity were analyzed by SDS-PAGE for the presence of *r*ARU at a molecular weight of approximately 102 kDa. Fractions containing a single band of *r*ARU were pooled. To further ensure purity the pooled solution was again passed over a Sepharose CL-6B column (25 ml media/100 ml protein solution). The solution containing purified *r*ARU was filtered sterilized by passage through a 22 μ filter and stored at 4°C. Purified *r*ARU along with samples from the steps of purification (lysate and dialyzed ammonium sulfate fraction) are shown in Fig. 5. The procedure typically yields approximately 100 mg *r*ARU per liter of *E. coli*/pRSETB-ARU-Km^r culture. A combined 6-liter batch yielded 0.850 liters of *r*ARU at 0.88 mg/ml for a total of 748 mg of *r*ARU or 125 mg/liter of culture. The amount of *r*ARU recovered represented 23% of the total soluble protein.

EXAMPLE 3

Synthesis of polysaccharide-*r*ARU conjugates.

Polysaccharides. Pneumococcal type 14 polysaccharide, Lot 40235-001, was manufactured by Lederle Laboratories, Pearl River, NY. *S. flexneri* type 2a O-specific polysaccharide and *E. coli* K1 polysaccharide were purified as described (Cohen, D. *et al. Lancet* 349:155-159 (1997); Devi *et al. Proc. Natl. Acad. Sci. USA* 88:7175-7179 (1991); Schneerson *et al. Infect. Immun.* 60:3528-3532 (1992)). All preparations had less than 1% protein and nucleic acid.

Chemicals. 1-ethyl-3-(3-dimethylaminopropyl) carboiimide, (EDC), succinic anhydride, MES (2-[N-morpholino]-thanesulfonic acid) hydrate, 2-[N-morpholino]-ethanesulfonic acid sodium salt), trinitrobenzenesulfonic acid (TNBS) and thimerosal,

were from Sigma Co., St. Louis, MO; adipic acid dihydrazide, cyanogen bromide and acetonitrile, from Sigma-Aldrich, Milwaukee, WI; CL-4b and CL-6B Sepharose, Sephadex G-50, from Pharmacia, Piscataway, NJ.

Analytical methods. The protein and saccharide components of the conjugates were assayed as described (Chu *et al. Infect. Immun.* 59:4450-4458 (1991)). Derivatization with adipic acid dihydrazide was measured by the trinitobenzene sulfonic acid assay (Chu *et al. Infect. Immun.* 59:4450-4458 (1991)). The extent of succinylation was measured indirectly by the reduction in amino groups of rARU using lysine as a standard (Fields R. *Biochem J.* 124:581-590 (1971); Pavliokova *et al. Infect. Immun.* 67:5526-5529 (1999)).

Succinylation of *r*ARU. Preliminary experiments defined the conditions that succinylated *r*ARU while retaining its antigenicity as measured by double immunodiffusion with goat anti-CDTA (Pavliakova *et al. Infect. Immun.* 67:5526-5529 (1989)). Succinic anhydride was added to *r*ARU at w/w of 1/10 at room temperature with mixing: the pH maintained at 7.2-7.5 with 0.5 M NaOH in a pH stat. After 20 minutes, the reaction mixture was passed through a 2.5 X 50 cm Sephadex G-50 column in 0.2 M NaCl and the void volume peak pooled and concentrated.

Conjugation of polysaccharides to *r*ARU and *r*ARU*succ*. Pneumococcal type 14 polysaccharide and *S. flexneri* type 2a O-specific polysaccharide were activated with cyanogen bromide, derivatized with adipic acid dihydrazide, and bound to *r*ARU or *r*ARU*succ* by water-soluble carboiimide condensation as described with the exception that the pH of the reactants was maintained with 0.1 MES, pH 6.0 (Chu *et al. Infect. Immun.* 59:4450-4458 (1991); Cohen, D. *et al. Lancet* 349:155-159 (1997); Schneerson *et al. Infect. Immun.* 60:3528-3532 (1992)). *E. coli* K1 polysaccharide was both derivatized with adipic acid dihydrazide and bound to *r*ARU or *r*ARU*succ* by treatment with EDC (Devi *et al. Proc. Natl. Acad. Sci. USA* 88:7175-7179 (1991)).

The composition of the adipic acid dihydrazide derivatized polysaccharides and of the conjugates is shown in Table 1. Note that low yields of conjugates, using *r*ARU as the carrier, were obtained with the pneumococcal type 14 and *S. fexneri* type 2a polysaccharides. We were unable to synthesize a conjugate of the K1 polysaccharide with *r*ARU.

TABLE 1. Composition of *Clostridium difficile* recombinant enterotoxin A (*r*ARU) conjugates of pneumococcal type 14 (Pn14), *Escherichia coli* K1 (group B meningococcal) capsular polysaccharide and *Shigella flexneri* type 2a O-specific polysaccharide.

Conjugate	Percent adipic hydrazide	*r*ARU/ succinate	*r*ARU/ polysaccharide (w/w)	Yield (%) polysaccharide	*r*ARU
Pn-14-*r*ARU	2.07	NA	0.52	10.4	5.2
Pn-14 *r*ARU*succ*	2.07	34.4	2.91	13.0	38.0
SF-*r*ARU	5.50	NA	1.56	1.4	2.1
SF-*r*ARU*succ*	5.50	38.3	2.36	20.0	51.4
K1-*r*ARU*succ*	3.8	41.2	3.23	13.3	43.0

NA - Not available

EXAMPLE 4.

Immune response to polysaccharide component of the conjugates.

Vaccination of mice. Female 5 weeks-old general purpose Swiss Albino mice at the NIH or outbred hsd/ICR mice (Harlan Sprague Derby, Inc., Indianapolis, IN) were injected subcutaneously with 0.1 ml containing 2.5 μg polysaccharide in the conjugate every 2 weeks. Mice (n=10) were exsanguinated 2 weeks after the first injection and 1 week after the second and third injections.

Serologic. IgG and IgM antibodies to *S. flexneri* type 2a LPS and to *E. coli* K1 polysaccharides were measured by ELISA as described (Chu *et al. Infect. Immun.* 59:4450-4458 (1991); Devi *et al. Proc. Natl. Acad. Sci. USA* 88:7175-7179 (1991)). IgG anti-pneumococcal type 14 polysaccharide were assayed by ELISA and total polysaccharide antibody by radioimmunoassay (RIA) and as described (Kayhty *et al. J. Infect. Dis*. 172:1273-1278 (1995); Schneerson *et al. Infect. Immun.* 60:3528-3532 (1992); Shiffman *et al. J. Immunol. Methods* 33:130-144 (1992)).

Pneumococcal type 14 (Pn14) antibodies (Table 2). Both conjugates (Pn14-*r*ARU and Pn14-*r*ARU*succ*) elicited statistically significant rises of IgG antibodies after the first and the second injections ($p<0.005$). The third injection of both conjugates elicited rises in IgG (4.38 to 6.41 EU for Pn14-*r*ARU and 6.10 to 9.76 EU for Pn14-*r*ARU*succ*) and IgM (4.82 to 7.57 for Pn14-*r*ARU and 6.16 to 8.54 for Pn14-*r*ARU*succ*) but these were not statistically significant. Pneumococcal type 14 polysaccharide alone elicits only trace levels of antibodies in mice (Schneerson *et al. Infect. Immun.* 60:3528-3532 (1992)). PBS did not elicit Pn14 antibodies.

TABLE 2. Serum pneumococcal antibodies (Pn14) elicited in mice by conjugates composed of *Clostridium difficle* recombinant toxin A repeating units (*r*ARU) alone or succinylated (*r*ARU*succ*) bound to pneumococcal type 14 polysaccharide.

	ELISA (Geometric mean and 25-75 centiles)					
	IgG			IgM		
Conjugate	First injection	Second injection	Third injection	First injection	Second injection	Third injection
Pn14-*r*ARU	0.90[a] 0.77-1.26	4.38[b] 1.97-11.6	6.41[c] 4.56-7.37	2.32 1.57-4.35	4.82 3.70-9.12	7.57 4.86-10.4
Pn-14-*r*ARU*succ*	0.71[d] 0.42-1.65	6.10[e] 3.55-7.40	9.76[f] 7.10-12.4	1.38 0.59-2.0	6.16 4.37-9.41	8.54 6.41-9.66

c.b *vs* 0.90, f,e *vs* 0.71 $p<0.005$; c *vs* b, f *vs* e, NS; f *vs* c, NS

6 wks-old mice were injected s.c. with 2.5 mg of pneumococcal type 14 polysaccharide as a conjugate at 2 wk intervals. Mice (n=10) were exsanguinated 2 wks after the 1st injection and 7 days after the 2nd and 3rd injections and their sera assayed for IgG and IgM anti-pneumococcal type 14 polysacchride by ELISA. A hyperimmune serum, arbitrarily assigned a value of 100 ELISA units (EU) was the reference.

The correlation coefficients between the geometric mean levels of conjugate-induced pneumococcal type 14 polysaccharide antibodies for all post-vaccination sera, as measured by ELISA and RIA, were statistically significant (Table 3).

TABLE 3. Comparison between conjugate-induced serum *Streptococcus pneumoniae* type 14 geometiric mean antibody levels measured by RIA and ELISA (IgG).

		Geometric mean			
Conjugate	Injection	RIA	ELISA	r=	p=
Pn14-*r*ARU	1st	723	0.90	0.73138	0.02
Pn14-*r*ARU	2nd	2232	4.38	0.97738	0.0001
Pn14-*r*ARU	3rd	3732	6.41	0.89505	0.0005
Pn14-*r*ARU*succ*	1st	682	0.71	0.94647	0.0001
Pn14-*r*ARU*succ*	2nd	3985	6.10	0.94233	0.0001
Pn14-*r*ARU*succ*	3rd	5725	9.76	0.88912	0.0006

Pneumococcal type 14 antibodies were measured by ELISA expressed as units and by RIA expressed as ng antibody nitrogen/ml serum.

Shigella fexneri type 2 a (SF) IgG LPS antibodies (Table 4). Both SF-*r*ARU and SF-*r*ARU*succ* elicited LPS antibodies after the second injection compared to prevaccination levels (p=0.001). Reinjection for the third time elicited a rise of IgG anti-LPS for both conjugates but was statistically significant only for SF-*r*ARU*succ* (2.48 vs 0.37, p=0.04). The SF IgG anti-LPS levels induced by the two conjugates were not statistically different.

Escherichia coli K1 (meningococcus group B) IgG antibodies. K1-*r*ARU*succ* elicited a significant rise in antibodies after all 3 injections: first injection (1.35 EU), second (12.4 vs 1.35, p=0.0001) and third (104 vs 12.4, p=0.002).

TABLE 4. Serum LPS antibodies elicited in mice by *Shigella flexneri* 2a O-specific polysaccharide (SF) bound to *Clostridium difficile* recombinant toxin A repeating units (*r*ARU) alone or succinylated (*r*ARU*succ*)

	ELISA (Geometric mean and 25-75th centiles)			
	IgG		IgM	
Immunogen	Second injection	Third injection	Second injection	Third injection
SF-*r*ARU	0.75 (0.40-1.43)	1.61 (1.13-3.38)	6.92 (4.85-12.2)	7.18 (2.74-18.2)
SF-*r*ARU*succ*	0.37 (0.03-1.63)	2.48 (1.35-5.14)	1.54 (0.18-54.5)	4.06 (1.74-8.77)

b vs a, p=0.04

6 wks-old mice were injected subcutaneously with 2.5 mg of *S. flexneri t*ype 2a O-specific polysaccharide alone or as a conjugate at 2 wk intervals. Mice (n=10) were exsanguinated 7 days after the second and third injections and their sera assayed for IgG anti-LPS by ELISA. A hyperimmune serum pool, arbitrarily assigned a value of 100 ELISA units (EU), served as a reference

EXAMPLE 5.

Immune response to the *r*ARU component of the conjugates.

Antibodies to *C. difficile* toxin A (CDTA). Antibodies to native toxin A were measured by ELISA, with toxin A isolated from *C. difficile* as the coating antigen, and by in-vitro neutralization of cytotoxicity (Lyerly *et al. Infect. Immun.* 35:1147-1150 (1982)). Human intestinal epithelial HT-29 cells (ATCC HTB 38) were maintained in 96 well plates with McCoy's 5A medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere. HT-29 cells were chosen because of their high sensitivity to CDTA probably because of the high density of the carbohydrate receptor on their surface. Serial 2-fold dilutions of sera were incubated with 0.4 μg/ml of CDTA for 30 min at room temperature. CDTA-serum mixtures were added to the wells at a final concentration of 20 ng of toxin A per well (about 200 times the minimal cytotoxic dose for HT-29 cells) in a final volume of 0.2 ml. The neutralization titer is expressed as the reciprocal of the highest dilution that completely neutralized cytotoxicity.

TABLE 5. Serum antibodies (mg/ml) to *Clostridium difficile* toxin A (CDTA) elicited in mice by recombinant enterotoxin A (*r*ARU) or polysaccharides bound to *r*ARU alone or succinylated (*r*ARU*succ*)

Conjugate	mg *r*ARU Injected	ELISA (Geometric mean and 25-75 centiles) First injection	Second injection	Third injection
*r*ARU*	6.94	ND	ND	717 (621-863)
Pn14-*r*ARU	1.29	3.70 (2.55-5.08)	80.1 (69.8-131)	194 (113-236)
Pn14*r*ARU *succ*	7.30	7.94 (5.21-11.3)	183 (146-175)	371 (274-463)
SF-*r*ARU	3.90	ND	433 (258-609)	613 (485-778)
SF-*r*ARUsucc	6.94	ND	191 (118-291)	518 (366-615)
SF-*r*ARU*	3.90	ND	ND	437 (372-547)
SF-*r*ARU*succ**	6.94	ND	ND	242 (172-443)
K1	8.08	10.7 (6.75-17.2)	84.9 (72.5-131)	390 (279-470)

183 vs 7.94 p=0.0001, 371 vs 183 p=0.0005, 80.1 vs 3.70 p=0.0001, 194 vs 80.1 p=0.007, 7.94 vs 3.70 p=0.01, 183 vs 80.1 p=0.004, 371 vs 194 p=0.01

*hsd/ICR mice. Remainder were NIH SA mice. ND (not done).

6 wks-old mice were injected SC with 2.5 mg of polysaccharide as a conjugate at 2 wk intervals. Groups of mice (n=10) were exsanguinated 7 days after each injection and their sera assayed for anti-CDTA by ELISA.

All 5 conjugates elicited high levels of anti-CDTA (194-613 μg/ml) (Table 5). Since the 2.5 μg immunizing dose of the conjugates was based on its polysaccharide content, the amount of *r*ARU injected was different for each conjugate. For example, on a protein weight basis, Pn14-*r*ARU, with 1.29 μg of *r*ARU, elicited 194 μg CDTA antibody/ml (150.3 μg Ab/μg rARU injected). In contrast, Pn14-*r*ARU*succ*, that contained 7.3 μg of *r*ARU per dose, elicited 371 μg CDTA antibody/ml (50.8 μg Ab/μg *r*ARU*succ* injected). Pn14-*r*ARU induced more anti-CDTA per μg *r*ARU than Pn14-*r*ARU*succ*, however, the total amount of anti-CDTA elicited by Pn14-*r*ARU*succ* was greater due to its higher content of *r*ARU. The difference between the levels of anti-CDTA elicited by Pn14-*r*ARU (194 μg CDTA antibody/ml) compared with Pn14-*r*ARU*succ* (371 μg CDTA antibody/ml) was significant.

SF-*r*ARU, containing 3.9 μg of *r*ARU, elicited 437 μg CDTA antibody/ml (112.0 μg Ab/μg *r*ARU injected) compared to 518 μg CDTA antibody/ml for SF-*r*ARU*succ* (34.9 μg Ab/μg *r*ARU*succ* injected). Although the specific immunogenic activity for the *r*ARU*succ* was lower than that of the *r*ARU in the SF conjugates, there was no statistical difference between the levels of CDTA antibody elicited by

the two conjugates (437 μg Ab/ml for SF-*r*ARU*succ* vs 242 μg Ab/ml for SF-*r*ARU).

K1-*r*ARU*succ*, that elicited 390 μg CDTA antibody/ml, had comparable specific immunogenic activity of its *r*ARU component (48 μg Ab/ml per μg *r*ARU*succ*).

EXAMPLE 6

CDTA neutralizing antibodies.

Individual sera obtained 7 days after the third injection of the conjugates were assayed individually for their neutralization of approximately 200 times the cytotoxic dose of CDTA on human intestinal epithelial HT-29 cells. All sera from the mice immunized with the conjugates had a neutralizing titer greater than or equal to 64. The geometric mean and range of neutralizing titers for each conjugate is shown in Table 6.

Conjugate-induced antibody levels approached or surpassed the neutralizing activity of an affinity-purified goat antibody, containing 0.5 mg/ml, that was raised against formalin inactivated CDTA.

TABLE 6. Serum neutralizing activity against the *in vitro* cytotoxicity for HT-29 cells of *Clostridium difficile* toxin A (CDTA)

Immunogen	mg Ab/ml (ELISA)	Reciprocol neutralization titer (GM and range)	
Pn14-*r*ARU	194	104	64-256
Pn14-*r*ARU*succ*	371	111	64-128
SF-*r*ARU	613	194	64-256
SF-*r*ARU*succ*	518	181	64-256
Goat antitoxin (0.5 mg/ml)*			128
PBS			0

Neutralizing titers were the highest serum dilution that completely inhibited the cytotoxicity of CDTA (20 ng/well) on HT-29 cells. The titers represent the geometric mean of sera from general purpose Swiss Albino mice (n=10) obtained 7 days after the 3rd injection. Anti-CDTA was measued by ELISA and the mean value expressed as mg Ab/ml serum.

* Affinity purified goat antibody

EXAMPLE 7

Protection against lethal challenge with CDTA (Table 7).

Hsd/ICR mice were injected with SF-*r*ARU, SF-*r*ARU*succ* or *r*ARU as described in EXAMPLE 4 above. One week after the third injection, the mice were challenged intraperitoneally with a lethal dose (150 ng) of CDTA. Almost all mice vaccinated with either conjugate or *r*ARU were protected. Based upon the amount of *r*ARU injected, *r*ARU and SF-*r*ARU elicited similar levels of anti-CDTA. As expected, SF-*r*ARU*succ* elicited lower levels of anti-CDTA than the other two immunogens but the recipients were comparably protected.

TABLE 7. Protection of mice against lethal challenge with 150 ng of *Clostridium difficile* toxin A (CDTA) [a] inducued by vaccination with polysaccharide-*r*ARU conjugates

Immunogen	mg *r*ARU injected	Survivals /total	CDTA antibodies (ELISA) [b]	Reciprocal neutralization titer [c]
*r*ARU	6.94	19/20	717 (621-863)	128-256
SF-*r*ARU	3.90	17/20	437 (372-547)	128-256
SF-*r*ARU*succ*	6.94	19/20	242 (172-443)	64-256
PBS	0	2/15	Not determined	<2

a Mice (hsd/ICR) injected I.P. with 150 ng of CDTA 7 days after the 3rd injection of *r*ARU or conjugate.

b Mean μg/ml antibody level (25-75 centiles) of sera used for pool (n=10) from each group bled 4 h before challenge with CDTA.

c Highest dilutions of sera (range) that completely neutralized the cytotoxicity of CDTA (20 ng/well) on HT-29 cells.

This invention has been described by a direct description and by examples. As noted above, the examples are meant to be only examples and not to limit the invention in any meaningful way. Additionally, one having ordinary skill in the art to which this invention pertains in reviewing the specification and claims which follow

would appreciate that there are equivalents to those claimed aspects of the invention. The inventors intend to encompass those equivalents within the reasonable scope of the claimed invention.

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5

CLAIMS

1. An immunogenic composition comprising a recombinant protein and a polysaccharide component, wherein said protein is encoded by a gene from a strain of *Clostridium difficile* and said polysaccharide component is isolated from a strain of a pathogenic microorganism or chemically synthesized.

2. The immunogenic composition of claim 1, wherein said protein is a toxin or fragment thereof.

3. The immunogenic composition of claim 1, wherein said polysaccharide component is a capsular polysaccharide or a lipopolysaccharide.

4. The immunogenic composition of claim 1, wherein said protein is toxin A or a fragment thereof.

5. The immunogenic composition of claim 4, wherein said protein comprises a recombinant amino acid sequence that includes the toxin A repeating units (*r*ARU) or a fragment thereof.

6. The immunogenic composition of claim 5, wherein said protein is a fusion protein.

7. The immonogenic composition of claim 1, wherein said protein is toxin B or a fragment threreof.

8. The immunogenic composistion of claim 7, wherein said protein comprises a recombinant amino acid sequence that includes the toxin B repeating units (*r*BRU) or a fragment thereof.

9. The immunogenic composition of claim 8, wherein said protein is a fusion protein.

10. The immunogenic composition of claim 1, wherein said immunogenic composition elicits in a mammalian host an immune response that is T-cell dependent.

11. The immunogenic composition of claim 1, wherein said immunogenic composition elicits in a mammalian host an immune response that is T-cell independent.

12. The immunogenic composition of claim 1, wherein said immunogenic composition elicits in a mammalian host an immune response that is both T-cell dependent and T-cell independent.

13. The immunogenic composition of claim 10 or 11 or 12, wherein said immune response is a cellular dependent immune response.

14. The immunogenic composition of claim 10 or 11 or 12, wherein said immune response results in a booster effect in said mammalian host.

15. The immunogenic composition of claim 10 or 11 or 12, wherein said immune response elicits a protective response to a strain of said pathogenic microorganism.

16. The immunogenic composition of claim 10 or 11 or 12, wherein said immunogenic composition elicits a humoral immune response in a mammalian host.

17. The immunogenic composition of claim 10 or 11 or 12, wherein said immunogenic composition elicits both a humoral immune response and a cellular dependent immune response in a mammalian host.

18. The immunogenic composition of claim 10 or 11 or 12, wherein said immune response elicits a protective response to a strain of a pathogenic microorganism.

19. The immunogenic composition of claim 18, wherein said strain of a pathogenic microorganism produces said polysaccharide *in vivo*.

20. The immunogenic composition of claim 19, wherein said polysaccharide is isolated from a strain of a pathogenic microorganism selected from the group consisting of strains of: *Streptococcus pneumoniae*; *Neisseria meningitidis*; *Escherichia coli*; and *Shigella*.

21. The immunogenic composition of claim 20, wherein said immune response elicits a protective response to a strain of a pathogenic microorganism selected from the group consisting of strains of: *Streptococcus pneumoniae*; *Neisseria meningitidis*; *Escherichia coli* and *Shigella*.

22. The immunogenic composition of claim 19, wherein said polysaccharide is isolated from a serotype of *Streptococcus pneumoniae*, selected from the group consisting of serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 25, and 33F.

23. The immunogenic composition of claim 19, wherein said polysaccharide is isolated from serotype 14 of *Streptococcus pneumoniae*.

24. The immunogenic composition of claim 18, wherein said immune response elicits a protective response to a strain of *Streptococcus pneumoniae*.

25. The immunogenic composition of claim 18, wherein said polysaccharide is isolated from a strain of *Shigella flexneri*, serotype 2a.

26. The immunogenic composition of claim 18, wherein said immune response elicits a protective response to a strain of *Shigella*.

27. The immunogenic composition of claim 18, wherein said polysaccharide is isolated from *Escherichia coli* K1.

28. The immunogenic composition of claim 19, wherein said pathogenic microorganism is group B meningococcus (*Neisseria meningitidis* serogroup B).

29. The immunogenic composition of claim 19, wherein said pathogenic microorganism is *Escherichia coli* K1.

30. The immunogenic composition of claim 19, wherein said polysaccharide selected from the group of: *Staphylococcus aureus*; coagulase-negative *Staphylococcus*; *Enterococcus* species; *Enterobacter* species; *Candida* species; group B *Streptococcus; Escherichia coli;* and *Pseudomonas* species.

31. The immunogenic composition of claim 19, wherein said immune response elicits a protective response to a strain of a nosocomial pathogenic microorganism selected from the group consisting of strains of: *Staphylococcus aureus*; coagulase-negative *Staphylococcus*; *Enterococcus* species; *Enterobacter* species; *Candida* species; group B *Streptococcus; Escherichia coli;* and *Pseudomonas* species.

32. The immunogenic composition of claim 19, wherein said polysaccharide is isolated from *Staphylococcus aureus* serogroup 5.

33. The immunogenic composition of claim 19, wherein said pathogenic microorganism is *Staphylococcus aureus* serogroup 5.

34. The immunogenic composition of claim 19, wherein said polysaccharide is isolated from *Staphylococcus aureus* serogroup 8.

35. The immunogenic composition of claim 19, wherein said pathogenic microorganism is *Staphylococcus aureus* serogroup 8.

36. An immunogenic composition comprising a recombinant protein and a polysaccharide component, wherein said protein is encoded by a gene isolated from a strain of *Clostridium difficile* and said polysaccharide is a polysaccharide isolated from a strain of a pathogenic microorganism or chemically synthesized and wherein said composition further comprises a pharmaceutically acceptable carrier.

37. A vaccine comprising the immunogenic composition of claim 36.

38. The vaccine of claim 37, wherein said vaccine is formulated for use in humans.

39. The vaccine of claim 37, wherein said vaccine is formulated for use in animals.

40. A method for producing an immunogenic composition, comprising

constructing a genetic sequence encoding a recombinant protein, wherein said genetic sequence is isolated from a strain of *Clostridium difficile*;

expressing said recombinant protein in a microbial host;

recovering said recombinant protein from a culture of said host;

conjugating said protein to a polysaccharide component, wherein said polysaccharide component is isolated from a pathogenic microorganism or chemically synthesized; and

recovering said conjugated protein and polysaccharide component.

41. The method of claim 40, wherein the expression of said genetic sequence is regulated by an inducible promoter operatively positioned upstream of said sequence and functional in said host.

42. The method of claim 40, wherein said microbial host is *Escherichia coli*.

43. The method of claim 42, wherein the recombinant protein is expressed at a level greater than about 10 mg/ml.

44. The method of claim 42, wherein the recombinant protein is expressed at a level greater than about 50 mg/liter of said culture.

45. The method of claim 42, wherein the recombinant protein is expressed at a level greater than about 100 mg/liter of said culture.

46. The method of claim 40, wherein said protein is greater than about 50kDa.

47. The method of claim 40, wherein said protein is greater than about 90kDa.

48. The method of claim 40, wherein said protein is recovered by ammonium sulfate precipitation followed by ion exchange chromatography.

49. The method of claim 40, wherein said protein is succinylated.

50. The method of claim 40, wherein said protein is conjugated to said polysaccharide component following a reaction of said protein and said polysaccharide component with 1, ethyl-3-(3-dimethylaminopropyl) carboiimide hydrochloride.

51. The method of claim 40, wherein said polysaccharide component is activated by cyanogen bromide.

52. The method of claim 40, wherein said polysaccharide is derivatized by adipic acid dihydrazide.

53. A recombinant genetic sequence comprising a gene encoding a protein from a strain of *Clostridium difficile*.

54. The recombinant sequence of claim 53, wherein said gene encodes toxin A or a fragment thereof.

55. The recombinant sequence of claim 54, wherein said gene encodes the toxin A repeating units (*r*ARU) or a fragment thereof.

56. The recombinant sequence of claim 53, wherein said gene encodes toxin B or a fragment thereof.

57. The recombinant sequence of claim 56, wherein said gene encodes the toxin B repeating units (*r*BRU) or a fragment thereof.

58. An expression vector comprising the genetic sequence of claim 53 and a gene that confers a selective phenotype upon a microbial host.

59. The expression vector of claim 58, wherein said selective phenotype is resistance to kanamycin.

60. A microbial host transformed with the expression vector of claim 58 or claim 59.

61. The use of the immunogenic composition of claim 1 for the production of antibodies for passive immune therapy against a strain of said pathogenic microorganism.

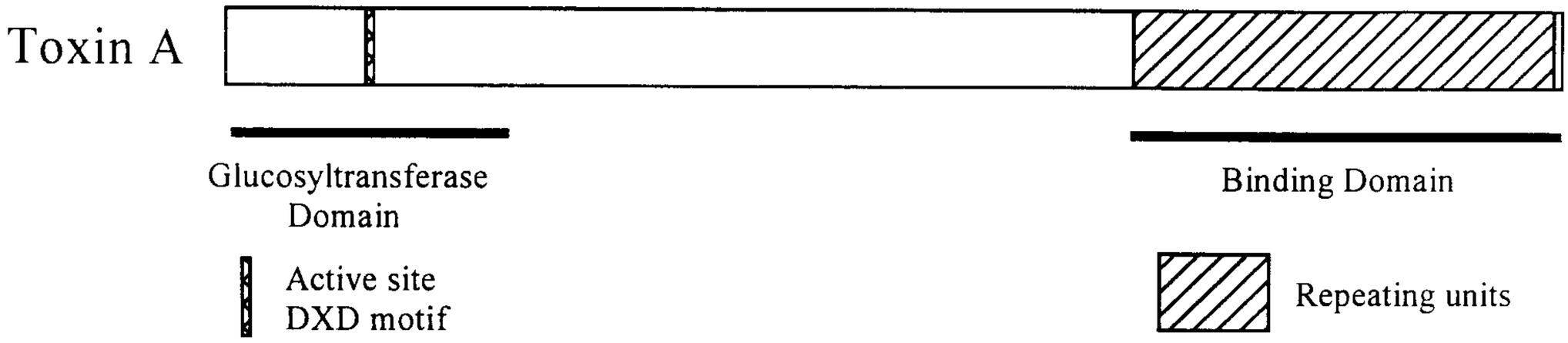

Toxin A
Glucosyltransferase Domain
Active site DXD motif
Binding Domain
Repeating units

Fig. 1

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tgcc*tga*

Fig. 2

DPIEFNLVTGWQTINGKKYYFDINTGAALTSYKIINGKHFY
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FLTLNGKKYYFDNNSKAVTGWRIINNEKYYFNPNNAIAAVG
LQVIDNNKYYFNPDTAIISKGWQTVNGSRYYFDTDTAIAFN
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Fig. 3

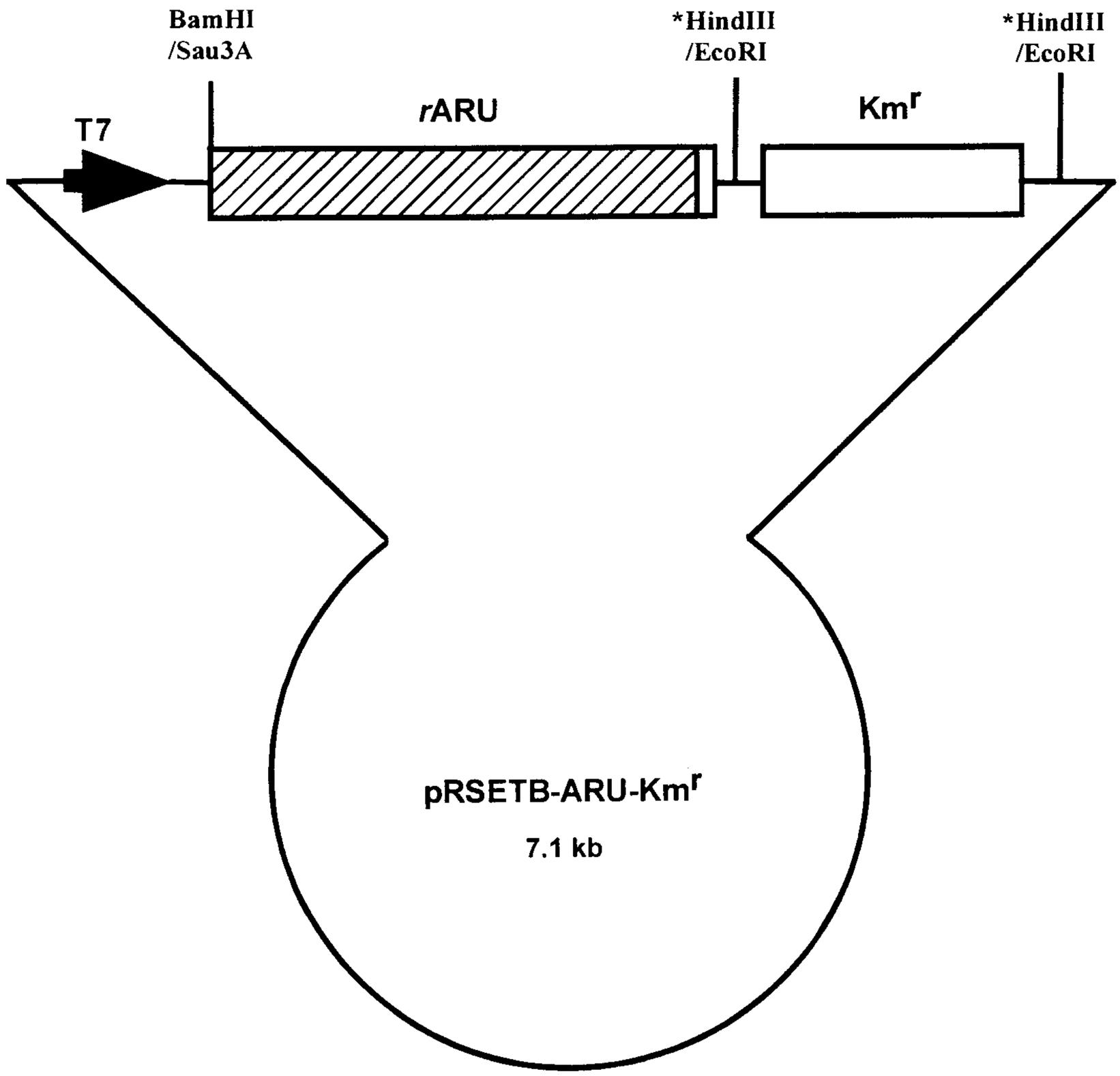

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7.1 kb

Fig. 4

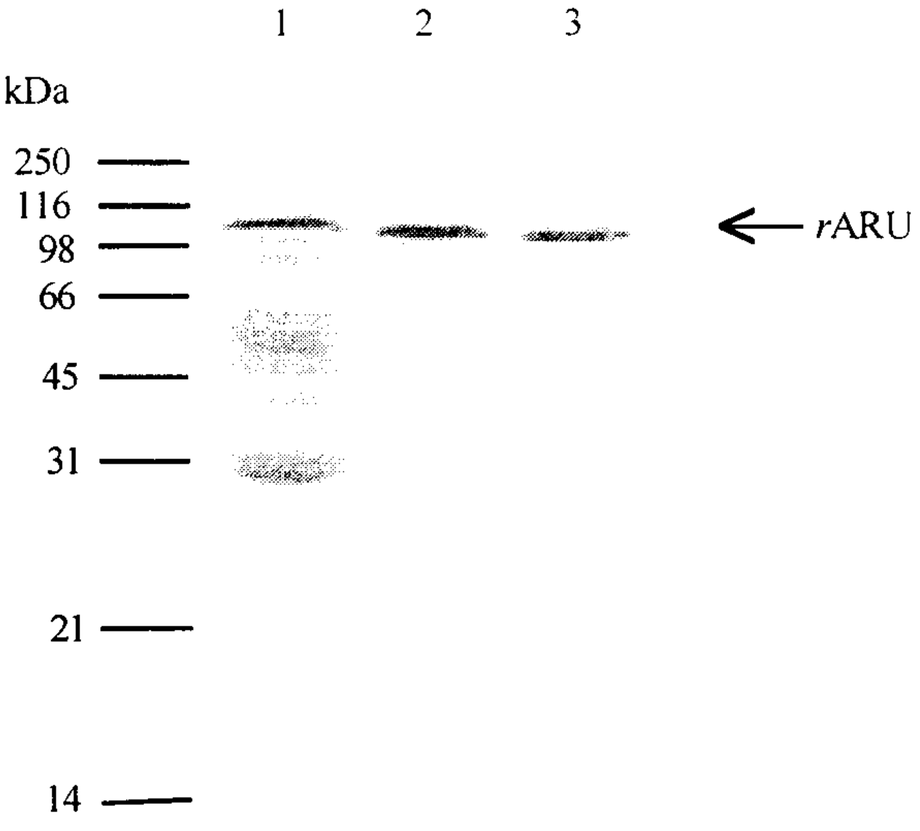
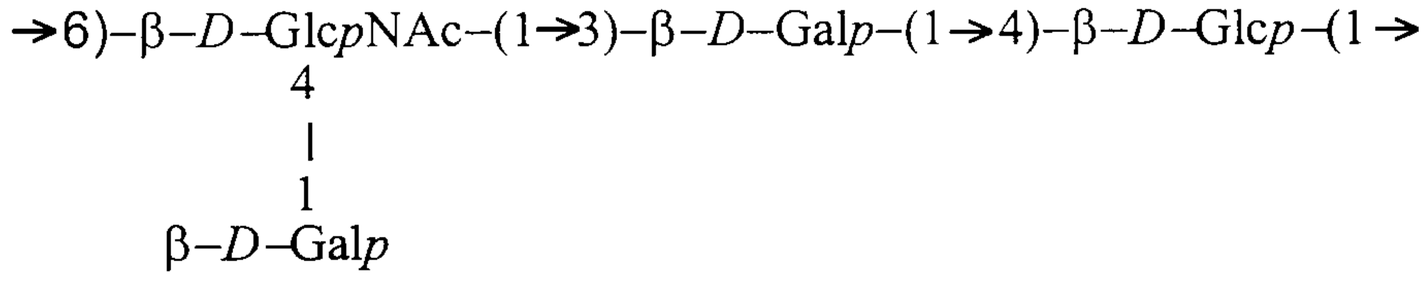
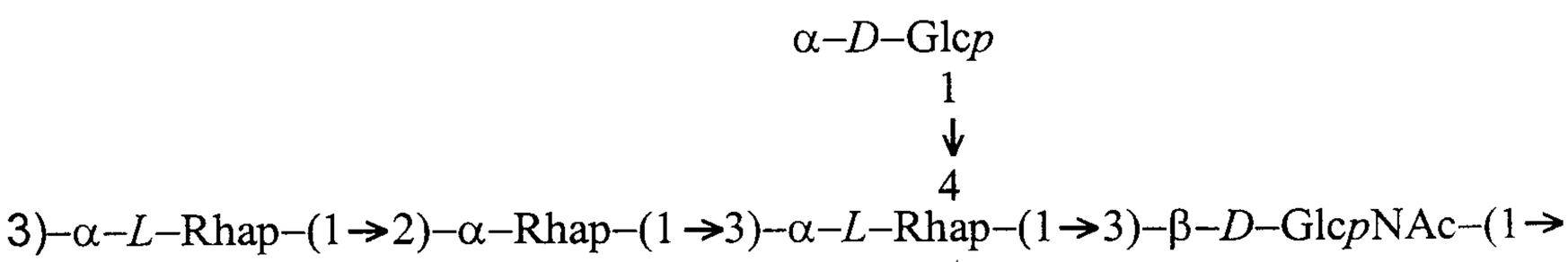


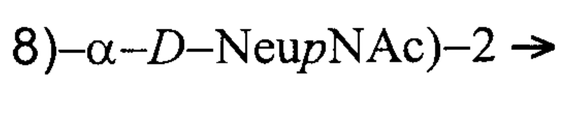
Fig. 5



Pneumococcus type 14

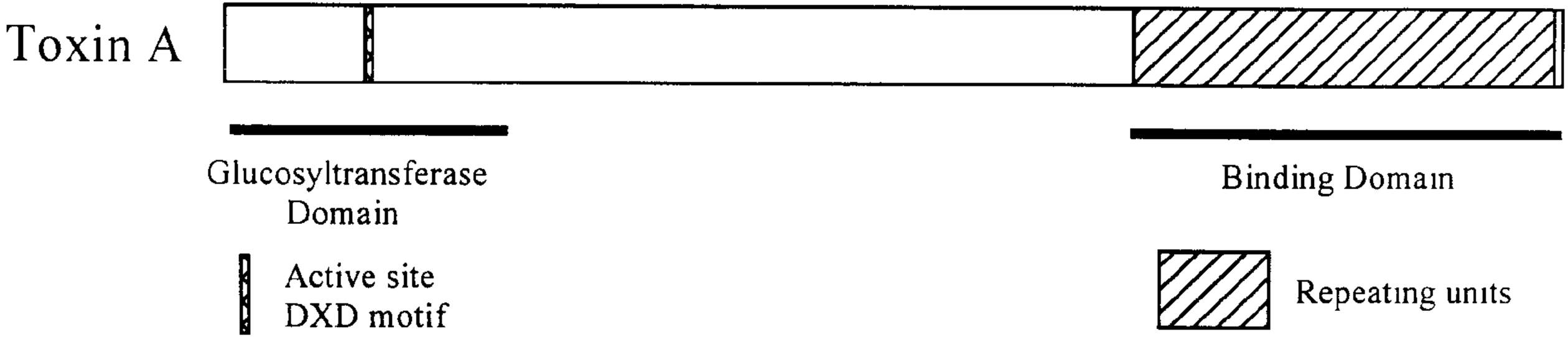


***Shigella flexneri* type 2a O-specific polysaccharide**



***Escherichia coli* K1**

Fig. 6


Toxin A
Glucosyltransferase Domain
Binding Domain
Active site DXD motif
Repeating units