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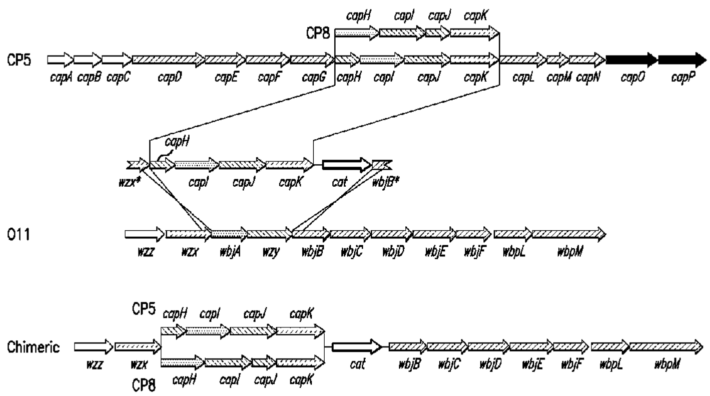
(54) **Titre : VACCINS DE BIOCONJUGUE DE BACTERIES GRAM POSITIF CAPSULAIRE**
(54) **Title: CAPSULAR GRAM-POSITIVE BACTERIA BIOCONJUGATE VACCINES**



(57) **Abrégé/Abstract:**
An embodiment of the present invention is directed to a novel S. aureus bioconjugate vaccine. More generally, the invention is directed to Gram-positive and other bioconjugate vaccines comprising: a protein carrier comprising an inserted nucleic acid consensus sequence; at least one polysaccharide such as a capsular Gram- positive polysaccharide linked to the consensus sequence; and, optionally, an adjuvant or pharmaceutically acceptable carrier. In a further aspect, the instant invention is directed to methods of producing Gram-positive and other bioconjugate vaccines. In another aspect, an N-glycosylated protein is provided that comprises one or more polysaccharides such as Gram-positive polysaccharides. The present invention is additionally directed to engineered prokaryotic organisms comprising nucleotide sequences encoding a glycosyltransferase of a first prokaryotic organism and a glycosyltransferase of a second prokaryotic organism. The invention further includes plasmids and prokaryotic cells transformed with plasmids encoding polysaccharides and enzymes which produce an N-glycosylated protein and/or bioconjugate vaccine. Further, the invention is directed to methods of inducing an immune response in a mammal comprising administering said bioconjugate vaccines.

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(54) **Title**: CAPSULAR GRAM-POSITIVE BACTERIA BIOCONJUGATE VACCINES

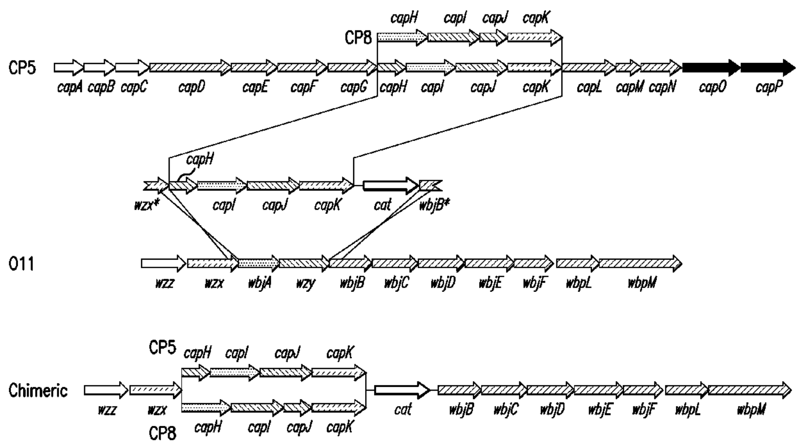

FIG.6

(57) **Abstract**: An embodiment of the present invention is directed to a novel *S. aureus* bioconjugate vaccine. More generally, the invention is directed to Gram-positive and other bioconjugate vaccines comprising: a protein carrier comprising an inserted nucleic acid consensus sequence; at least one polysaccharide such as a capsular Gram-positive polysaccharide linked to the consensus sequence; and, optionally, an adjuvant or pharmaceutically acceptable carrier. In a further aspect, the instant invention is directed to methods of producing Gram-positive and other bioconjugate vaccines. In another aspect, an N-glycosylated protein is provided that comprises one or more polysaccharides such as Gram-positive polysaccharides. The present invention is additionally directed to engineered prokaryotic organisms comprising nucleotide sequences encoding a glycosyltransferase of a first prokaryotic organism and a glycosyltransferase of a second prokaryotic organism. The invention further includes plasmids and prokaryotic cells transformed with plasmids encoding polysaccharides and enzymes which produce an *N*-glycosylated protein and/or bioconjugate vaccine. Further, the invention is directed to methods of inducing an immune response in a mammal comprising administering said bioconjugate vaccines.

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CAPSULAR GRAM-POSITIVE BACTERIA BIOCONJUGATE VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 61/332,170, filed May 6, 2010.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format.

BACKGROUND OF THE INVENTION

[0004] Vaccines have been one of the great public health inventions of modern medicine and have saved millions of lives. Immunizations have been proven to be an ideal means to prevent and control infections. Each year vaccines prevent up to 3 million deaths and 750,000 children are saved from disability. (Global Alliance for Vaccines and Immunization - Press Releases (March 11, 2006) *at* www.gavialliance.org/media_centre/press_releases/2006_03_09_en_pr_queenrania_delhi.php). In 1999 the CDC declared immunizations the number one public health achievement of the 20^{th} century (Ten great public health achievements-United States, 1900-1999. MMWR Morb Mortal Wkly Rep 48:241-3 (April 2, 1999)). Some bacteria like those causing tetanus or diphtheria produce a toxin that is largely responsible for the disease. This toxin can be used in a detoxified form as vaccine. However, for most bacteria there is no single toxin that can be used to develop a vaccine.

[0005] Among the most successful vaccines are surface polysaccharides of bacterial pathogens like *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* conjugated to carrier proteins. These bacteria are surrounded by a capsule, which promotes microbial virulence and resistance to phagocytic killing, as well as preventing them from desiccation.

[0006] Bacterial polysaccharides can elicit a long-lasting immune response in humans if they are coupled to a protein carrier that contains T-cell epitopes. This concept was elaborated 80 years ago (Avery, O. T., and W. F. Goebel. 1929. Chemo-immunological studies on conjugated carbohydrate-proteins. II Immunological specificity of synthetic sugar-proteins. J. Exp. Med. 50:521-533), and proven later for the polysaccharide of *Haemophilus influenza* type B (HIB) coupled to the protein carrier diphtheria toxin (Anderson, P. 1983. Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugates of oligosaccharides of the type b capsule with the nontoxic protein CRM197. Infect Immun 39:233-8; Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins. 1980. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J Exp Med 152:361-76). This glycoconjugate was also the first conjugated vaccine to be licensed in the USA in 1987 and introduced into the US infant immunization schedule shortly thereafter. Besides HIB, conjugated vaccines were successfully used against the encapsulated human pathogens *N. meningitidis* and *S. pneumoniae*. Routine use of these vaccines has resulted in decreased nasopharyngeal colonization, as well as infection. Currently approximately ~25% of the global vaccine market comprises conjugated vaccines.

[0007] Gram-positive bacteria have a cell membrane that is surrounded by capsular polysaccharides. *Staphylococcus* is one such Gram-positive bacterium.

[0008] *Staphylococcus aureus* causes infection. *S. aureus* is an opportunistic bacterial pathogen responsible for a diverse spectrum of human diseases. Although *S. aureus* may colonize mucosal surfaces of normal humans, it is also a major cause of wound infections and has the invasive potential to induce severe infections, including osteomyelitis, endocarditis, and bacteremia with metastatic complications (Lowy, F. D. 1998. *Staphylococcus aureus* infections.

New Engl J Med 339:520-32). *S. aureus* is one of the most common agents implicated in ventilator-associated pneumonia, and it is an important and emerging cause of community-acquired pneumonia, affecting previously healthy adults and children lacking predisposing risk factors (Kollef, M. H., A. Shorr, Y. P. Tabak, V. Gupta, L. Z. Liu, and R. S. Johannes. 2005. Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. Chest 128:3854-62; Shorr, A. F. 2007. Epidemiology and economic impact of meticillin-resistant *Staphylococcus aureus*: review and analysis of the literature. Pharmacoeconomics 25:751-68).

[0009] *S. aureus* is the second most common cause of nosocomial bacteremia, and methicillin-resistant *S. aureus* (MRSA) strains account for more than 50% of all infections in intensive care units in the U.S. *S. aureus* infections within the hospital and in the community are increasing. MRSA strains were isolated from 2% of staphylococcal infections in 1974 and from 63% of staphylococcal infections in 2004. Many of the nosocomial MRSA strains are multi-drug resistant, and even methicillin-sensitive strains can be deadly. A recent report using population-based, active case finding revealed that 94,360 invasive MRSA infections occurred in the U.S. in 2005, and that the majority of these (58%) occurred outside of the hospital (Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 298:1763-71). In this analysis, more Americans died from MRSA (>18,000 deaths) in 2005 than from AIDS.

[0010] *S. aureus* USA100, also known as the New York/Japan clone, is an MRSA strain that represents the predominant U.S. hospital-acquired MRSA strain (McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover. 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41:5113-20).

[0011] Epidemiologic analyses indicate that *S. aureus* causes approximately 2 million clinical infections each year in the U.S. alone (Fridkin, S. K., J. C. Hageman, M. Morrison,

L. T. Sanza, K. Como-Sabetti, J. A. Jernigan, K. Harriman, L. H. Harrison, R. Lynfield, and M. M. Farley. 2005. Methicillin-resistant *Staphylococcus aureus* disease in three communities. N Engl J Med 352:1436-44; King, M. D., B. J. Humphrey, Y. F. Wang, E. V. Kourbatova, S. M. Ray, and H. M. Blumberg. 2006. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med 144:309-17; Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, S. K. Fridkin, and M. I. for the Active Bacterial Core surveillance. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 298:1763-1771). Not only are *S. aureus* infections increasing in number, but the resistance of *S. aureus* to antibiotics is also on the increase. MRSA accounts for 40%-60% of nosocomial *S. aureus* infections in the U.S., and many of these strains are multi-drug resistant. Notorious as a major source of nosocomial infections, *S. aureus* has recently taken on a new role in causing an escalating number of community-acquired infections in non-hospitalized persons without predisposing risk factors. Virulent community-associated MRSA (CA-MRSA) strains are becoming more prevalent across the U.S. and Europe, and their dissemination has been observed globally (Baggett, H. C., T. W. Hennessy, K. Rudolph, D. Bruden, A. Reasonover, A. Parkinson, R. Sparks, R. M. Donlan, P. Martinez, K. Mongkolrattanothai, and J. C. Butler. 2004. Community-onset methicillin-resistant *Staphylococcus aureus* associated with antibiotic use and the cytotoxin Panton-Valentine leukocidin during a furunculosis outbreak in rural Alaska. J Infect Dis 189:1565-73; Gilbert, M., J. MacDonald, D. Gregson, J. Siushansian, K. Zhang, S. Elsayed, K. Laupland, T. Louie, K. Hope, M. Mulvey, J. Gillespie, D. Nielsen, V. Wheeler, M. Louie, A. Honish, G. Keays, and J. Conly. 2006. Outbreak in Alberta of community-acquired (USA300) methicillin-resistant *Staphylococcus aureus* in people with a history of drug use, homelessness or incarceration. Canad Med Assoc J 175:149-54; Kazakova, S. V., J. C. Hageman, M. Matava, A. Srinivasan, L. Phelan, B. Garfinkel, T. Boo, S. McAllister, J. Anderson, B. Jensen, D. Dodson, D. Lonsway, L. K. McDougal, M. Arduino, V. J. Fraser, G. Killgore, F. C. Tenover, S. Cody, and D. B. Jernigan. 2005. A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. N Engl J Med 352:468-75).

[0012] Not only has *S. aureus* resistance to methicillin become more common, but numerous isolates with reduced susceptibility to vancomycin have been reported. Seven clinical isolates of *S. aureus* that carry *vanA* and are fully resistant to vancomycin have been isolated in the U.S. These isolates are also methicillin resistant (Chang, S., D. M. Sievert, J. C. Hageman, M. L. Boulton, F. C. Tenover, F. P. Downes, S. Shah, J. T. Rudrik, G. R. Pupp, W. J. Brown, D. Cardo, and S. K. Fridkin. 2003. Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. New Engl J Med 348:1342-7). Because *S. aureus* cannot always be controlled by antibiotics and MRSA isolates are becoming increasingly prevalent in the community, additional control strategies, such as a vaccine, are sorely needed.

[0013] *S. aureus* capsular polysaccharides are involved in infection. Many virulence factors contribute to the pathogenesis of staphylococcal infections, including surface-associated adhesions, secreted exoproteins and toxins, and immune evasion factors (Foster, T. J. 2005. Immune evasion by staphylococci. Nature Reviews Microbiology 3:948-58). Like many invasive bacterial pathogens, *S.aureus* produces a capsular polysaccharide (CP) (FIG. 4) that enhances its resistance to clearance by host innate immune defenses. Most clinical isolates of *S.aureus* are encapsulated, and serotype 5 and 8 strains predominate (Arbeit, R. D., W. W. Karakawa, W. F. Vann, and J. B. Robbins. 1984. Predominance of two newly described capsular polysaccharide types among clinical isolates of *Staphylococcus aureus*. Diagn Microbiol Infect Dis 2:85-91). The type 5 (CP5) and type 8 (CP8) capsular polysaccharides have similar trisaccharide repeating units comprised of *N*-acetyl mannosaminuronic acid (ManNAcA), *N*-acetyl L-fucosamine (L-FucNAc), and *N*-acetyl D-fucosamine (D-FucNAc) (Jones, C. 2005. Revised structures for the capsular polysaccharides from *Staphylococcus aureus* types 5 and 8, components of novel glycoconjugate vaccines. Carbohydr Res 340:1097-106). CP5 and CP8 are serologically distinct, and this can be attributed to differences in the linkages between the sugars and in the sites of O-acetylation (FIG. 4).

[0014] Previous studies have correlated *S. aureus* capsule production with resistance to *in vitro* phagocytic uptake and killing (Fattom, A., R. Schneerson, S. C. Szu, W. F. Vann, J. Shiloach, W. W. Karakawa, and J. B. Robbins. 1990. Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A. Infect Immun 58:2367-74; Thakker, M., J.-S.

Park, V. Carey, and J. C. Lee. 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun 66:5183-5189; Watts, A., D. Ke, Q. Wang, A. Pillay, A. Nicholson-Weller, and J. C. Lee. 2005. *Staphylococcus aureus* strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. Infect Immun 73:3502-11). Human neutrophils phagocytose capsule-negative mutants in the presence of nonimmune serum with complement activity, whereas encapsulated isolates require both capsule-specific antibodies and complement for optimal opsonophagocytic killing (Bhasin, N., A. Albus, F. Michon, P. J. Livolsi, J.-S. Park, and J. C. Lee. 1998. Identification of a gene essential for O-acetylation of the *Staphylococcus aureus* type 5 capsular polysaccharide. Mol Microbiol 27:9-21; Thakker, M., J.-S. Park, V. Carey, and J. C. Lee. 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun 66:5183-5189; Watts, A., D. Ke, Q. Wang, A. Pillay, A. Nicholson-Weller, and J. C. Lee. 2005. *Staphylococcus aureus* strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. Infect Immun 73:3502-11). Nilsson *et al.* (Nilsson, I.-M., J. C. Lee, T. Bremell, C. Ryden, and A. Tarkowski. 1997. The role of staphylococcal polysaccharide microcapsule expression in septicemia and septic arthritis. Infect Immun 65:4216-4221) reported that peritoneal macrophages from mice phagocytosed significantly greater numbers of a CP5-negative mutant compared to the parental strain Reynolds. Once phagocytosed, the CP5-positive strain survived intracellularly to a greater extent than the mutant strain. Cunnion *et al.* (Cunnion, K. M., J. C. Lee, and M. M. Frank. 2001. Capsule production and growth phase influence binding of complement to *Staphylococcus aureus*. Infect Immun 69:6796-6803) compared opsonization of isogenic *S. aureus* strains and demonstrated that the CP5-positive strain bound 42% less serum complement (C') than the acapsular mutant.

[0015] *S. aureus* vaccine development conventionally has involved the capsule as a target. Vaccine design for protection against staphylococcal disease is complicated by the protean manifestations and clinical complexity of *S. aureus* infections in humans. Many *S. aureus* vaccine candidates have been investigated in animal models of infection, but it has been reported that only two immunization regimens have completed phase III clinical trials (Schaffer, A. C., and J. C. Lee. 2008. Vaccination and passive immunisation against *Staphylococcus aureus*. Int J Antimicrob

Agents 32 Suppl 1:S71-8). The first vaccine is based on the two capsular polysaccharides (CPs) (FIG. 4) that are most prevalent among clinical strains of *S. aureus*. Fattom *et al.* (Fattom, A.R. Schneerson, S. C. Szu, W. F.Vann, J. Shiloach, W. W. Karakawa and J. B. Robbins. 1990. Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin. Infect Immun 58: 2367-74) conjugated the serotype 5 (CP5) and serotype 8 (CP8) polysaccharides to nontoxic recombinant *P. aeruginosa* exoprotein A (rEPA). The conjugate vaccines were immunogenic in mice and humans, and they induced opsonic antibodies that showed efficacy in protecting rodents from lethality and from nonlethal staphylococcal infection (Fattom, A.R. Schneerson, S. C. Szu, W. F.Vann, J. Shiloach, W. W. Karakawa and J. B. Robbins. 1990. Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin. Infect Immun 58: 2367-74; Fattom, A., R. Schneerson, D. C. Watson, W. W. Karakawa, D. Fitzgerald, I. Pastan, X. Li, J. Shiloach, D. A. Bryla, and J. B. Robbins. 1993. Laboratory and clinical evaluation of conjugate vaccines composed of *S. aureus* type 5 and type 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exoprotein A. Infect Immun 61:1023-32; Fattom, A. I., J. Sarwar, A. Ortiz, and R. Naso. 1996. A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. Infect Immun 64:1659-65; Lee, J. C., J. S. Park, S. E. Shepherd, V. Carey, and A. Fattom. 1997. Protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide in a modified model of endocarditis in rats. Infect Immun 65:4146-51). Passive immunization studies have indicated that both CP5- and CP8-specific antibodies significantly reduce infection in a murine model of *S. aureus* mastitis (Tuchscherr, L. P., F. R. Buzzola, L. P. Alvarez, J. C. Lee, and D. O. Sordelli. 2008. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. Infect Immun 76:5738-44). The combined CP5- and CP8-conjugate vaccine was shown to be safe in humans and elicited antibodies that showed opsonophagocytic activity.

[0016] *S. aureus* vaccine development has also involved surface proteins as a target. The second *S. aureus* clinical vaccine trial was based on the protective efficacy of antibodies to staphylococcal adhesions in preventing staphylococcal infections. *S. aureus* clumping factor A is a cell wall-anchored protein that is surface expressed, mediates staphylococcal adherence to fibrinogen (Foster, T. J., and M. Hook. 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 6:484-8), and promotes the attachment of *S. aureus* to biomaterial surfaces (Vaudaux, P. E., P. Francois, R. A. Proctor, D. McDevitt, T. J. Foster, R. M. Albrecht, D. P. Lew, H. Wabers, and S. L. Cooper. 1995. Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infection & Immunity 63:585-90), blood clots, and damaged endothelial surfaces (Moreillon, P., J. M. Entenza, P. Francioli, D. McDevitt, T. J. Foster, P. Francois, and P. Vaudaux. 1995. Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. Infection & Immunity 63:4738-43). The fibrinogen-binding domain of ClfA is located within region A of the full-length protein (McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster. 1995. Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. Molecular Microbiology 16:895-907). ClfA plays an important role in *S. aureus* binding to platelets, an interaction that is critical in animal models of catheter-induced staphylococcal endocarditis (Sullam, P. M., A. S. Bayer, W. M. Foss, and A. L. Cheung. 1996. Diminished platelet binding in vitro by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. Infection & Immunity 64:4915-21).

[0017] Nanra *et al.* reported that antibodies to ClfA induced opsonophagocytic killing of *S. aureus in vitro* (Nanra, J. S., Y. Timofeyeva, S. M. Buitrago, B. R. Sellman, D. A. Dilts, P. Fink, L. Nunez, M. Hagen, Y. V. Matsuka, T. Mininni, D. Zhu, V. Pavliak, B. A. Green, K. U. Jansen, and A. S. Anderson. 2009. Heterogeneous in vivo expression of clumping factor A and capsular polysaccharide by *Staphylococcus aureus*: Implications for vaccine design. Vaccine 27:3276-80). Furthermore, mice immunized with a recombinant form of the binding region A of ClfA showed reductions in arthritis and lethality induced by *S. aureus* (Josefsson, E., O. Hartford, L. O'Brien, J. M. Patti, and T. Foster. 2001. Protection against experimental *Staphylococcus aureus* arthritis by vaccination with clumping factor A, a novel virulence determinant. Journal of Infectious Diseases 184:1572-80). Passive immunization experiments were performed in rabbits given a human polyclonal immunoglobulin preparation that contained elevated levels of antibodies specific for ClfA (Vernachio, J., A. S. Bayer, T. Le, Y. L. Chai, B. Prater, A. Schneider, B. Ames, P. Syribeys, J. Robbins, J. M. Patti, J. Vernachio, A. S. Bayer, T. Le, Y.-L. Chai, B. Prater, A. Schneider, B. Ames, P. Syribeys, J. Robbins, and J. M. Patti. 2003. Anti-clumping factor A immunoglobulin reduces the duration of methicillin-resistant *Staphylococcus aureus* bacteremia in an experimental model of infective endocarditis. Antimicrobial Agents & Chemotherapy 47:3400-6). The combination therapy resulted in better bacterial clearance from the blood of rabbits with catheter-induced *S. aureus* endocarditis than did vancomycin treatment alone. In addition, passive transfer of ClfA-specific antibodies significantly reduced infection in a murine model of *S. aureus* mastitis (Tuchscherr, L. P., F. R. Buzzola, L. P. Alvarez, J. C. Lee, and D. O.Sordelli. 2008. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. Infect Immun 76: 5738-44).

[0018] A phase III clinical trial was reportedly designed to protect against late-onset sepsis in 2000 low birth weight, premature neonates. The infants received up to four administrations of Veronate, a human immunoglobulin preparation pooled from donors with elevated antibody titers against ClfA and SdrG. Despite the promising results from a similar phase II clinical trial, this prophylactic therapy resulted in no reduction in the frequency of staphylococcal infections in the neonates (DeJonge, M., D. Burchfield, B. Bloom, M. Duenas, W. Walker, M. Polak, E. Jung, D. Millard, R. Schelonka, F. Eyal, A. Morris, B. Kapik, D. Roberson, K. Kesler, J. Patti, and S. Hetherington. 2007. Clinical trial of safety and efficacy of INH-A21 for the prevention of nosocomial staphylococcal bloodstream infection in premature infants. J Pediatr 151:260-5).

[0019] It has been shown that protein glycosylation occurs, but rarely does so naturally, in prokaryotic organisms. On the other hand, *N*-linked protein glycosylation is an essential and conserved process occurring in the endoplasmic reticulum of eukaryotic organisms. It is important for protein folding, oligomerization, stability, quality control, sorting and transport of secretory and membrane proteins (Helenius, A., and Aebi, M. (2004). Roles of *N*-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. 73, 1019-1049). Protein glycosylation has a profoundly favorable influence on the antigenicity, the stability and the half-life of a protein. In addition, glycosylation can assist the purification of proteins by chromatography, e.g. affinity chromatography with lectin ligands bound to a solid phase interacting with glycosylated moieties of the protein. It is therefore established practice to produce many glycosylated proteins recombinantly in eukaryotic cells to provide biologically and pharmaceutically useful glycosylation patterns.

[0020] Conjugate vaccines have been successfully used to protect against bacterial infections. The conjugation of an antigenic polysaccharide to a protein carrier is required for protective memory response, as polysaccharides are T-cell independent antigens. Polysaccharides have been conjugated to protein carriers by different chemical methods, using activation reactive groups in the polysaccharide as well as the protein carrier. (Qian, F., Y. Wu, O. Muratova, H. Zhou, G. Dobrescu, P. Duggan, L. Lynn, G. Song, Y. Zhang, K. Reiter, N. MacDonald, D. L. Narum, C. A. Long, L. H. Miller, A. Saul, and G. E. Mullen. 2007. Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. Vaccine 25:3923-3933; Pawlowski, A., G. Kallenius, and S. B. Svenson. 2000. Preparation of pneumococcal capsular polysaccharide-protein conjugates vaccines utilizing new fragmentation and conjugation technologies. Vaccine 18:1873-1885; Robbins, J. B., J. Kubler-Kielb, E. Vinogradov, C. Mocca, V. Pozsgay, J. Shiloach, and R. Schneerson. 2009. Synthesis, characterization, and immunogenicity in mice of *Shigella sonnei* O-specific oligosaccharide-core-protein conjugates. Proc Natl Acad Sci U S A 106:7974-7978).

[0021] Conjugate vaccines can be administered to children to protect them against bacterial infections and can provide a long lasting immune response to adults. Constructs of the invention have been found to generate an IgG response in animals. It is believed that the polysaccharide (i.e. sugar residue) triggers a short-term immune response that is sugar-specific. Indeed, the human immune system generates a strong response to specific polysaccharide surface structures of bacteria, such as O-antigens and capsular polysaccharides. However, as the immune response to polysaccharides is IgM dependent, the immune system develops no memory. The protein carrier that carries the polysaccharide, however, triggers an IgG response that is T-cell dependent and that provides long lasting protection since the immune system develops memory. For this reason, in developing a vaccine, it is advantageous to develop it as a protein carrier - polysaccharide conjugate.

[0022] Prokaryotic organisms rarely produce glycosylated proteins. However, it has been demonstrated that a bacterium, the food-borne pathogen *Campylobacter jejuni*, can glycosylate its proteins (Szymanski, *et al.* (1999). Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. Mol. Microbiol. 32, 1022-1030). The machinery required for glycosylation is encoded by 12 genes that are clustered in the *pgl* locus. Disruption of glycosylation affects invasion and pathogenesis of *C. jejuni* but is not lethal as in most eukaryotic organisms (Burda P. and M. Aebi, (1999). The dolichol pathway of *N*-linked glycosylation. Biochim Biophys Acta 1426(2):239-57). It has been shown that the pgl locus is responsible for N-linked protein glycosylation in *Campylobacter* and that it is possible to reconstitute the N-glycosylation of *C. jejuni* proteins by recombinantly expressing the *pgl* locus and acceptor glycoprotein in *E. coli* at the same time (Wacker, M., D. Linton, P. G. Hitchen, M. Nita-Lazar, S. M. Haslam, S. J. North, M. Panico, H. R. Morris, A. Dell, B. W. Wren, and M. Aebi. 2002. *N*-linked glycosylation in *C. jejuni* and its functional transfer into *E. coli*. Science 298:1790-3).

[0023] The *N*-linked protein glycosylation biosynthetic pathway of *Campylobacter* has significant similarities to the polysaccharide biosynthesis pathway in bacteria (Bugg, T. D., and P. E. Brandish. 1994. From peptidoglycan to glycoproteins: common features of lipid-linked oligosaccharide biosynthesis. FEMS Microbiol Lett 119:255-62). Based on the knowledge that antigenic polysaccharides of bacteria and the oligosaccharides of *Campylobacter* are both synthesized on the carrier lipid, undecaprenyl pyrophosphate (UndPP), the two pathways were combined in *E. coli* (Feldman, M. F., M. Wacker, M. Hernandez, P. G. Hitchen, C. L. Marolda, M. Kowarik, H. R. Morris, A. Dell, M. A. Valvano, and M. Aebi. 2005. Engineering *N*-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A 102:3016-21). It was demonstrated that PglB does not have a strict specificity for the lipid-linked sugar substrate. The antigenic polysaccharides assembled on UndPP are captured by PglB in the periplasm and transferred to a protein carrier (Feldman, M. F., M. Wacker, M. Hernandez, P. G. Hitchen, C. L. Marolda, M. Kowarik, H. R. Morris, A. Dell, M. A. Valvano, and M. Aebi. 2005. Engineering *N*-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A 102:3016-21; Wacker, M., M. F. Feldman, N. Callewaert, M. Kowarik, B. R. Clarke, N. L. Pohl, M. Hernandez, E. D.

Vines, M. A. Valvano, C. Whitfield, and M. Aebi. 2006. Substrate specificity of bacterial oligosaccharyltransferase (OTase) suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proc Natl Acad Sci U S A 103:7088-93). It was shown that *Campylobacter* PglB transfers a diverse array of UndPP linked oligosaccharides if they contain an N-acetylated hexosamine at the reducing terminus (Wacker *et al.* (2006)), allowing conjugation of an antigenic polysaccharide to a protein of choice through an N-glycosidic linkage. While this may provide a theoretical basis for production of conjugated vaccines *in vivo*, many difficult challenges need to be overcome in order to realize this theoretical possibility.

[0024] Based on this previous discovery that *C. jejuni* contains a general *N*-linked protein glycosylation system, *E. coli* had been modified to include the *N*-linked protein glycosylation machinery of *C. jejuni*. In this way, glycosylated forms of proteins native to *C. jejuni* in an *E. coli* host were produced. It had been further shown that this process could be used to produce glycosylated proteins from different origins in modified *E. coli* host for use as vaccine products. Production by *E. coli* is advantageous because large cultures of such modified *E. coli* hosts can be produced which produce large quantities of useful vaccine.

[0025] Using this process to produce a glycosylated protein in a modified *E. coli* host for use as a vaccine product for *S. aureus* encounters problems that have been perceived to be insurmountable. First, *E. coli* is a Gram-negative bacterium and its saccharide biosynthesis pathways differ greatly from those of a Gram-positive bacterium, such as *S. aureus*, after the polymerization step. In addition, it would have been infeasible to genetically engineer *E. coli* to produce the *S. aureus* capsular polysaccharide directly consistent with previous technologies. For example, *S. aureus* is a Gram positive organism and its capsule synthesis is associated with cell envelope structure and construction of the cellular hull. The capsule producing biosynthetic machinery is specifically designed to arrange the capsular polysaccharide (PS) on the outside of the cell and its cell wall. It would have been extremely difficult, for at least the reason that it would be highly resource-intensive, to produce this capsule in a modified *E. coli* organism, because the cell envelope of *E. coli* is constructed in a fundamentally different way. The biosynthetic machinery for capsule assembly from PS precursor would be non-functional due to the different environment. Whereas the *S. aureus* capsule must transit a single membrane, in *E. coli* there is an additional membrane which needs to be crossed to reach the final location of an authentic capsule. Furthermore, as the *S. aureus* capsule is very large, it was believed to be infeasible to make a large capsule like the *S. aureus* capsule between the two membranes of *E. coli*.

[0026] The principle that enzymes from different organisms can work together has been shown before (e.g. Rubires, X., F. Saigi, N. Pique, N. Climent, S. Merino, S. Alberti, J. M. Tomas and M. Regue. 1997. A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the rfb-50 mutation of *Escherichia coli* K-12 derivatives. J. Bacteriol 179(23): 7581-6). However, it is believed that no modified LPS polysaccharide from a Gram-positive organism has previously been produced in a Gram-negative organism.

BRIEF SUMMARY OF THE INVENTION

[0027] We have now surprisingly discovered a novel *S. aureus* bioconjugate vaccine. This novel *S. aureus* vaccine is based on the novel and unexpected discovery that an oligo- or polysaccharide of a prokaryote having one Gram strain can glycosylate a protein in a host prokaroyte having a different Gram strain. Further novel and unexpected features of the invention include without limitation the embodiments set forth below.

[0028] More generally, the present invention is directed to a bioconjugate vaccine, such as a Gram-positive vaccine, comprising a protein carrier comprising an inserted nucleic acid consensus sequence; at least one oligo- or polysaccharide from a bacterium such as a Gram-positive bacterium linked to the consensus sequence, and, optionally, an adjuvant. Further, the invention is directed to a Gram-positive bacteria vaccine, such as an *S. aureus* vaccine, or other bacteria vaccine, made by a glycosylation system using a modified LPS biosynthetic pathway, which comprises the production of a modified capsular polysaccharide or LPS.

[0029] The instant invention is additionally directed to a recombinant *N*-glycosylated protein comprising a protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and at least one oligo- or polysaccharide from a bacterium such as a Gram-positive bacterium linked to said consensus sequence.

[0030] The present is furthermore directed to a combination of a modified capsular polysaccharide of *S. aureus* with a protein antigen from the same organism by *N*-glycosidic linkage.

[0031] The invention is further directed to host prokaryotic organisms comprising a nucleotide sequence encoding one or more glycosyltransferase of a first prokaryotic species, such as a Gram-positive species; one or more glycosyltransferases of a different prokaryotic species, such as a Gram-negative species; a nucleotide sequence encoding a protein; and a nucleotide sequence encoding an OTase. The invention is additionally directed to an engineered host prokaryotic organism comprising an introduced nucleotide sequence encoding glycosyltransferases native only to a Gram-positive prokaryotic organism; a nucleotide sequence encoding a protein; and a nucleotide sequence encoding an OTase.

[0032] The invention is furthermore directed to methods of producing a bioconjugate vaccine in a host prokaryotic organism comprising nucleic acids encoding one or more glycosyltransferases of a first prokaryotic species, such as a Gram-positive species, for example, *S. aureus*; one or more glycosyltransferases of a second prokaryotic species, a protein; and an OTase. In addition, the present invention is directed to the production of bioconjugate vaccines by producing in Gram-negative bacteria modified capsular polysaccharides, which can be transferred to lipid A core by WaaL and/or linked to a carrier of choice by the OTase.

[0033] The invention is further directed to methods of producing glycosylated proteins in a host prokaryotic organism comprising nucleotide sequence encoding glycosyltransferases native to a first prokaryotic organism and also encoding glycosyltransferases native to a second prokaryotic organism that is different from the first prokaryotic organism. The present invention is additionally directed to the production of proteins *N*-glycosylated with capsular polysaccharides of Gram-positive bacteria, which are synthesized by a combination of different glycosyltransferases from different organisms. The invention is furthermore directed to the production of glycosylated proteins in a host prokaryotic organism comprising an introduced nucleotide sequence encoding glycosyltransferases native only to a Gram-positive prokaryotic organism.

[0034] The instant invention is moreover directed to plasmids, such as, plasmids comprising one or more of SEQ. ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. The invention also includes plasmids comprising one or more of SEQ. ID NO: 6; SEQ. ID NO: 7; SEQ. ID NO: 8 and SEQ. ID NO: 16. The invention also relates to plasmids comprising one or more of SEQ. ID NO: 10; SEQ. ID NO: 11; and SEQ. ID NO: 12. Moreover, the invention is directed to plasmids comprising one or more of SEQ. ID NO: 13; SEQ. ID NO: 15; SEQ. ID NO: 15; SEQ. ID NO: 17; SEQ ID NO: 18; SEQ. ID NO: 19; SEQ. ID NO: 20; SEQ. ID NO: 21 and SEQ. ID NO: 27.

[0035] The invention is additionally directed to transformed bacterial cells, such as, for example, bacterial cells transformed with a plasmid comprising one or more of SEQ. ID NO. 2; SEQ. ID NO: 3; SEQ. ID NO: 4; SEQ. ID NO: 17; SEQ. ID NO: 18; SEQ. ID NO: 19 and SEQ. ID NO: 20; SEQ. ID NO: 21; and SEQ. ID NO: 27. The instant invention is further directed to a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO: 5; SEQ. ID NO: 8; SEQ. ID NO: 9; SEQ. ID NO: 10; SEQ. ID NO: 11; SEQ. ID NO: 12; SEQ. ID NO: 13; SEQ. ID NO: 14; SEQ. ID NO: 15 and SEQ. ID NO: 16.

[0036] The instant invention is further directed to a method of inducing an immune response against an infection caused by Gram-positive and other bacteria in a mammal. In one embodiment, the method comprises administering to said mammal an effective amount of a pharmaceutical composition comprising: protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and one or more oligo- or polysaccharides, the one or more oligo- or polysaccharides being the same or different as another of the one or more oligo- or polysaccharides, from a Gram-positive bacterium linked to said consensus sequence.

[0037] In another aspect, the invention features a method of identifying a target polysaccharide for use in glycosylating a protein with said target polysaccharide, in whole or in part. Said glycosylated protein comprising the target polysaccharide can be used, for example, in vaccine compositions. In one embodiment, the method of identifying a target polysaccharide includes: identifying a Gram-positive bacterium, such as *S. aureus*, as a target; identifying a first repeating unit of a polysaccharide produced by said Gram-positive bacterium comprising at least three monomers; identifying a polysaccharide produced by a bacterium of a Gram-negative species comprising a second repeating unit comprising two of the same monomers as said first repeating unit.

[0038] The present invention is also directed to a method for modifying a bacterium of a first bacterial species such as a Gram-negative species. In one embodiment, the method includes: identifying a first repeating unit of a polysaccharide of a Gram-positive species, such as *S. aureus*, comprising three monomers; identifying a polysaccharide produced by a bacterium of a second Gram-negative species comprising another repeating unit comprising two of the same monomers of the first repeating unit; inserting into said bacterium of a first Gram-negative species one or more nucleotide sequences encoding glycosyltransferases that assemble a trisaccharide comprising: a) said second repeating unit; and b) a monomer of said first repeating unit not present in said second repeating unit; inserting a nucleotide sequence encoding a protein; and inserting a nucleotide sequence encoding an OTase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **FIG. 1** depicts a pathway for the wzx/wzy-dependent O-antigen biosynthesis, exemplified by the *P. aeruginosa* O11 O-antigen biosynthesis. Protein names putatively responsible for the presented reactions are indicated above or below the arrows, including uridine diphosphate (UDP) and uridine monophosphate (UMP).

[0040] **FIG. 2** depicts a proposed pathway for the engineered *S. aureus* capsular polysaccharide serotype 5 (CP5) biosynthesis in *E. coli*. The enzymes provided by the O-antigen cluster of *P. aeruginosa* O11 are indicated as in FIG. 1. Enzymes from *S. aureus* CP5 are indicated as Cap5 (compare to FIG. 6). WecB and WecC are *E. coli* enzymes required for the production of UDP-ManNAcA. Other depicted proteins and enzymes include uridine diphosphate (UDP), uridine monophosphate (UMP), and coenzyme A (CoA).

[0041] **FIG. 3** depicts a proposed pathway for the engineered *S. aureus* capsular polysaccharide serotype 8 (CP8) biosynthesis. Gene names are indicated by arrows (compare to FIG. 1, 2, and 6). UDP, UMP: uridine diphosphate, uridine monophosphate. CoA: coenzyme A.

[0042] **FIG. 4** depicts the structural overlap of capsular *S. aureus* and *P. aeruginosa* O-antigen Repeating Unit (RU) Structures.

[0043] **FIG. 5A** depicts the SDS-PAGE analysis of the elongation of the incomplete O11 O-antigen RU (repeating unit) by *S. aureus* enzymes.

[0044] **FIG. 5B** depicts the immunodetection of the elongation of the incomplete O11 O-antigen RU by *S. aureus* enzymes.

[0045] **FIG. 6** depicts a strategy in an embodiment of the invention for the construction of the chimeric O11/CP5 and O11/CP8 gene clusters.

[0046] **FIG. 7A** depicts polymerized CP5 LPS of an embodiment of the invention detected in *E. coli* lipid extracts.

[0047] **FIG. 7B** depicts polymerized CP8 LPS of an embodiment of the invention detected in *E. coli* lipid extracts.

[0048] **FIG. 8A** depicts recombinant CP5 LPS production of an embodiment of the invention analyzed by SDS-PAGE and stained by silver in dependence of antibiotic resistance gene on the pLAFR plasmid containing the chimeric cluster in W3110 Δ*wecA* cells.

[0049] **FIG. 8B** depicts recombinant CP5 LPS production of an embodiment of the invention analyzed by SDS PAGE, stained by silver and immunodetection in dependence of antibiotic resistance gene on the pLAFR plasmid containing the chimeric cluster in W3110 Δ*wecA* cells.

[0050] **FIG. 9** depicts recombinant CP5 LPS production of an embodiment of the invention analyzed SDS PAGE and by immunodetection in dependence of promoter in front of the chimeric cluster in W3110 Δ*wecA* cells.

[0051] **FIG. 10A** shows the results of HPLC analysis of an embodiment of recombinant RU of CP5 of the present invention produced using the chimeric CP5 cluster (SEQ ID: 2).

[0052] **FIG. 10B** shows the results of HPLC analysis of an embodiment of recombinant RU of CP8 of the present invention produced using a chimeric CP8 cluster lacking the *cap8I* polymerase.

[0053] **FIG. 11A** shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP5 cluster of the present invention in *E. coli* eluting at 37 minutes seen in FIG. 10A.

[0054] **FIG. 11B** shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP5 cluster of the present invention in *E. coli* eluting at 40 minutes seen in FIG. 10A.

[0055] **FIG. 11C** shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP8 cluster of the present invention in *E. coli* eluting at 32 minutes seen in FIG. 10B.

[0056] **FIG. 11D** shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP8 cluster of the present invention in *E. coli* eluting at 38 minutes seen in FIG. 10B.

[0057] **FIG. 11E** shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP8 cluster of the present invention in *E. coli* eluting at 45 minutes seen in FIG. 10B.

[0058] **FIG. 11F** shows the results of HPLC analysis of an embodiment of glycan structure optimization.

[0059] **FIG.11G** (including **FIG. 11G-1**) presents the results of HPLC analysis of the full CP5 glycan repertoire present on UndPP in *E. coli* cells in an embodiment of the present invention.

[0060] **FIG. 11H** presents the results of HPLC analysis of deacetylated CP5 glycans and RU homogeneity in an embodiment of the invention.

[0061] **FIG. 11I** provides the results of HPLC analysis of the CP8 glycan repertoire present on UndPP in *E. coli* cells in an embodiment of the present invention.

[0062] **FIG. 11J** shows HPLC results, in an embodiment of the present invention, of deacetylation of CP8 glycans and RU homogeneity.

[0063] **FIG. 11K** presents HPLC results showing reduction in RU polymerization and increase in LLO induced by co-expression of *wzz*O7 with the CP8 chimeric cluster in an embodiment of the present invention.

[0064] **FIG. 12** shows the results of SDS-PAGE analysis of Ni^{2+} affinity chromatography purified EPA-CP5 bioconjugate from cells in embodiments of the present invention without and with the *S. aureus* flippase gene *cap5K* (SEQ ID NO: 2 and 3).

[0065] **FIG. 13A** presents analysis of CP5-EPA bioconjugate according to an embodiment of the present invention purified by Ni^{2+} affinity chromatography and anionic exchange chromatography.

[0066] **FIG. 13B** depicts M/Z masses found for the glycosylation site in trypsinized peptide DNNNSTPTVISHR N-glycosidically linked to the O-acetylated RU mass (m/z=2088 ($[M+H]^+$)) according to an embodiment of the present invention. The inset illustrates the RU structure attached to the peptide.

[0067] **FIG. 13C** depicts M/Z masses found for the glycosylation site in trypsinized peptide DQNR N-glycosidically linked to the O-acetylated RU mass (m/z=1165 ($[M+H]^+$)) according to an embodiment of the present invention. The inset illustrates the RU structure attached to the peptide.

[0068] **FIG. 13D** depicts an analysis of Ni^{2+} affinity chromatography and anionic exchange chromatography purified CP8-EPA bioconjugate according to an embodiment of the present invention.

[0069] **FIG. 13E** depicts purified CP5-EPA bioconjugate from cells containing either 3 (left) or 2 plasmids (right lane) for glycoconjugate production according to an embodiment of the present invention.

[0070] **FIG. 13F** depicts analysis of Ni^{2+} affinity chromatography purified CP8-EPA bioconjugate according to an embodiment of the present invention.

[0071] **FIG. 14A** presents High Mass MALDI analysis of a purified CP5-EPA bioconjugate of an embodiment of the invention produced using the 3 plasmid system from FIG. 13A.

[0072] **FIG. 14B** shows characterization by size exclusion chromatography of CP5-EPA bioconjugate of an embodiment of the invention produced using the 3 plasmid system from FIG. 13A.

[0073] **FIG. 14C** shows the SDS PAGE analysis and immunodetection of purified CP5-Hla bioconjugate according to an embodiment of the present invention.

[0074] **FIG. 14D** presents the results of purified CP5-AcrA bioconjugate according to an embodiment of the present invention.

[0075] **FIG. 14E** presents the results of purified CP5-ClfA bioconjugate according to an embodiment of the present invention.

[0076] **FIG. 15A** depicts the specific anti CP5 antibodies raised in mice by CP5-EPA bioconjugate according to an embodiment of the present invention.

[0077] **FIG. 15B** depicts the specific anti CP5 antibodies raised in rabbit by CP5-EPA bioconjugate according to an embodiment of the present invention.

[0078] **FIG. 16A** illustrates *in vitro* opsonophagocytic activity (on *S. aureus* Reynolds) of CP5 specific antibodies raised by immunization of rabbits with CP5-EPA according to an embodiment of the present invention.

[0079] **FIG. 16B** illustrates *in vitro* opsonophagocytosis activity (on *S. aureus* USA 100) of CP5 specific antibodies raised by immunization of rabbits with CP5-EPA according to an embodiment of the present invention.

[0080] **FIG. 17A** depicts the results of passive immunization using anti CP5-EPA antibodies, according to an embodiment of the present invention, in mice challenged i.p. with ~$3.6.10^7$ CFU of *S. aureus* strain Reynolds.

[0081] **FIG. 17B** depicts the results of passive immunization using anti CP5-EPA antibodies, according to an embodiment of invention, in mice injected with 2 mg CP5-EPA IgG.

[0082] **FIG. 17C** depicts the results of passive immunization using anti CP5-EPA antibodies, according to an embodiment of the invention, in mice injected with 300 µg CP5-EPA IgG

[0083] **FIG. 18** depicts the results of an active immunization assay using different doses of CP5-EPA as vaccine according to an embodiment of the present invention and the mouse bacteremia model for challenge.

DETAILED DESCRIPTION OF THE INVENTION

[0084] According to an embodiment of the present invention, an LPS polysaccharide from a Gram-positive organism has now been shown to be produced in a Gram-negative organism. We believe that this is a novel result that represents an important and significant departure from the prior art.

[0085] Nucleic acids within the scope of the invention are exemplified by the nucleic acids of the invention contained in the Sequence Listing. Any nucleic acid encoding an immunogenic component, or portion thereof, which is capable of expression in a host cell, can be used in the present invention. The following sequence descriptions are provided to facilitate understanding of certain terms used throughout the application and are not to be construed as limiting embodiments of the invention.

[0086] SEQ ID NO: 1 depicts pLAFR1 (Gene Bank Accession AY532632.1) containing the O11 O-antigen sequence from *P. aeruginosa* PAO103 in the EcoRI site, complementary strand (partially from Gen Bank Accession AF236052).

[0087] SEQ ID NO: 2 depicts pLAFR1 containing the CP5 chimeric cluster, corresponding to the pLAFR1-O11 with the *cap5HIJ* genes replacing *wbjA-wzy* by homologous recombination. The inserted sequence also contains a *cat* cassette for selection of homologous recombined clones.

[0088] SEQ ID NO: 3 depicts pLAFR1 containing the CP5 chimeric cluster with the *cap5K* flippase gene, corresponding to the pLAFR1-O11 with the *cap5HIJ* genes replacing *wbjA-wzy* by homologous recombination and the *cap5K* cloned between *cap5J* and the *cat* cassette.

[0089] SEQ ID NO: 4 depicts pLAFR1 containing the CP8 chimeric cluster including a flippase gene, corresponding to the pLAFR1-O11 with the *cap8KHIJ* genes replacing *wbjA-wzy*. The inserted sequence also contains a *cat* cassette for selection of homologous recombined clones.

[0090] SEQ ID NO: 5 depicts an expression plasmid for Hla H35L production. The ORF encoding Hla H35L is cloned into *Nde*I/*Sac*I in pEC415.

[0091] SEQ ID NO. 6 depicts the expression plasmid for Hla-H35L site 202 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, , a glycosite around amino acid position 202, and a C-terminal HIS-tag. This construct is cloned into NheI/SalI on pEC415.

[0092] SEQ ID NO: 7 depicts the expression plasmid for Hla-H35L site 238 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 238, and a C-terminal HIS-tag. The above mentioned construct is cloned into NheI/SalI on pEC415.

[0093] SEQ ID NO: 8 depicts the expression plasmid for Hla-H35L site 272 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 272, and a C-terminal HIS-tag. The above mentioned construct is cloned into NheI/SalI on pEC415.

[0094] SEQ ID NO: 9 depicts an expression plasmid for ClfA production. The gene was chemically synthesized and cloned into the *Nde*I/ *Sac*I in pEC415 expression vector.

[0095] SEQ ID NO: 10 depicts the expression plasmid for ClfA site 290 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 290, and a C-terminal HIS-tag. The above mentioned construct is cloned into NheI/SalI on pEC415.

[0096] SEQ ID NO: 11 depicts the expression plasmid for ClfA site 327 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 327, and a C-terminal HIS-tag. The above mentioned construct is cloned into NheI/SalI on pEC415.

[0097] SEQ ID NO: 12 depicts the expression plasmid for ClfA site 532 production The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 532, and a C-terminal HIS-tag. The above mentioned construct is cloned into NheI/SalI on pEC415.

[0098] SEQ ID NO: 13 depicts the amino acid sequence of recombinant, genetically detoxified EPA with a signal sequence and two glycosylation sites at positions 260 and 402.

[0099] SEQ ID NO: 14 depicts the amino acid sequence of recombinant, genetically detoxified EPA without signal sequence and two glycosylation sites at positions 241 and 383.

[0100] SEQ ID NO: 15 depicts the ORF encoding AcrA cloned via NheI/SalI into pEC415.

[0101] SEQ ID NO: 16 depicts the expression plasmid for Hla-H35L site 130 production. The ORF encodes an N-terminal DsbA signal peptide from *E. coli*, a glycosite around amino acid position 130, and a C-terminal HIS-tag. The above mentioned construct is cloned NheI/SalI into pEC415.

[0102] SEQ ID NO: 17 depicts CP5 producing gene cluster with *cap5K* flippase followed by a *pglB* expression cassette consisting of the intergene DNA sequence between galF and wbqA of *E. coli* serotype O121 and the pglB ORF. The insert is cloned in the EcoRI site of pLAFR1.

[0103] SEQ ID NO: 18 depicts CP8 producing gene cluster with *cap8K* flippase followed by a *pglB* expression cassette consisting of the intergene DNA sequence between *galF* and *wbqA* of *E. coli* serotype O121 and the *pglB* ORF. The insert is cloned in the EcoRI site of pLAFR1.

[0104] SEQ ID NO: 19 depicts CP8 producing gene cluster with cap8K flippase followed by a *pglB* expression cassette consisting of the intergene DNA sequence between *galF* and *wbqA* of *E. coli* serotype O121 and the *pglB* ORF, in addition this sequence has the gene for *wzz* of the *E. coli* serovar O7 cloned into SfaAI/BspTI, i.e. between *wzx* of *Pseudomonas aeruginosa* O11 and cap8H. The insert is cloned in the EcoRI site of pLAFR1.

[0105] SEQ ID NO: 20 depicts an expression plasmid for EPA and wzz. The backbone is pACT3 in which the resistance cassette was replaced (kanamycin for chloranphenicol)

[0106] SEQ ID NO: 21 depicts *wzz* of *E. coli* serotype O7 cloned in pext21 Eco/Sal.

[0107] SEQ ID NO: 22 depicts a peptide sequence set forth in the Examples.

[0108] SEQ ID NO: 23 depicts a peptide sequence set forth in the Examples.

[0109] SEQ ID NO: 24 depicts a protein consensus sequence, D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline.

[0110] SEQ ID NO: 25 depicts a glycosylation site.

[0111] SEQ ID NO: 26 depicts a glycosylation site.

[0112] SEQ ID NO: 27 depicts an expression plasmid containing the *pglB* ORF cloned in EcoRI/BamHI sites.

[0113] Descriptions of terms and abbreviations appear below as used in the specification and consistent with the usages known to one of ordinary skill in the art. The descriptions are provided to facilitate understanding of such terms and abbreviations and are not to be construed as limiting embodiments of the invention.

[0114] AcrA refers to a glycoprotein from C. jejuni.

[0115] Active immunization refers to the induction of immunity (antibodies) after exposure to an antigen.

[0116] APCs refers to antigen presenting cells.

[0117] Amp refers to ampicillin.

[0118] Bacteremia refers to the presence of viable bacteria in the circulating blood.

[0119] C' refers to complement.

[0120] CapA is an enzyme proposed to be a chain length determinant in *S. aureus* CP5.

[0121] CapB is an enzyme proposed to be a regulator of polysaccharide chain length in *S. aureus* CP5.

[0122] CapC is an enzyme proposed to encode a transporter protein in *S. aureus* CP5.

[0123] CapD an enzyme having 4,6 dehydratase activity and converts the precursor UDPGlcNAc to UDP-2-acetamido-2,6 dideoxy-D-xylo-4-hexulose in *S. aureus* CP5.

[0124] CapE is a 4,6-dehydratase 3,5-epimerase catalyzing the epimerization of UDP-D-GlcNAc to UDP-2-acetamido-2, 6-dideoxy-D-*lyxo*-4-hexulose in *S. aureus* CP5.

[0125] CapF is a reductase, catalyzes the reduction form UDP-2-acetamido-2, 6-dideoxy-D-*lyxo*-4-hexulose to UDP-L-6dTalNAc in *S. aureus* CP5.

[0126] CapG is a 2-Epimerase, catalyzes the epimerization form UDP-L-6dTalNAc to UDP-LFucNAc in *S. aureus* CP5.

[0127] CapH in *S. aureus* CP5 is an O-acetyltransferase.

[0128] CapH in CP8 is a transferase similar to CapI from *S. aureus* CP5.

[0129] CapI in *S. aureus* CP5 is a glycosyltransferase which catalyzes the transfer of UDP-ManNAcA into carrier lipid-D-FucNAc-L-FucNAc producing carrier lipid-D-FucNAc-L-FucNAc-ManNAcA.

[0130] CapI in CP8 is a polymerase which is similar to CapJ in *S. aureus* CP5.

[0131] CapJ in *S. aureus* CP5 is a polymerase.

[0132] CapJ in CP8 is an O-acetyltransferase similar to CapH in *S. aureus* CP5.

[0133] CapK in *S. aureus* CP5 is a flippase.

[0134] CapK in *S. aureus* CP8 is a flippase similar to the CapK in CP5.

[0135] CapL is a transferase which catalyzes the transfer of UDP-L-FucNAc onto D-FucNAc-carrier lipid producing carrier lipid-D-FucNAc-L-FucNAc in *S. aureus* CP5.

[0136] CapM is a transferase which catalyzes the transfer of UDP-D-FucNAc on to carrier lipid producing carrier lipid-D-FucNAc in *S. aureus* CP5.

[0137] CapN is a 4-reductase which catalyzes the reduction from UDP-2-acetamido-2, 6-dideoxy-D-xylo-4-hexulose.to UDP-D-FucNAc in *S. aureus* CP5.

[0138] CapO is a dehydrogenase which catalyzes the conversion of UDP-D-ManNAc into UDP-ManNAcA in *S. aureus* CP5.

[0139] CapP is a 2-epimerase which catalyzes the epimerization of UDP-D-GlcNAc to UDP-D-ManNAc in *S. aureus* CP5.

[0140] CFU refers to Colony formation unit.

[0141] ClfA refers to *S. aureus* clumping factor A, a cell wall-anchored protein.

[0142] Conjugate vaccine refers to a vaccine created by covalently attaching a polysaccharide antigen to a carrier protein. Conjugate vaccine elicits antibacterial immune responses and immunological memory. In infants and elderly people a protective immune response against polysaccharide antigens can be induced if these antigens are conjugated with proteins that induce a T-cell dependent response.

[0143] Consensus sequence refers to a sequence of amino acids, -D/E - X - N - Z - S/T- wherein X and Z may be any natural amino acid except Proline, within which the site of carbohydrate attachment to *N*-linked glycoproteins is found.

[0144] Capsular polysaccharide, in its naturally occurring form, refers to a thick, mucous-like layer of polysaccharide, is water soluble and commonly acidic. Naturally-occurring capsular polysaccharides consist of regularly repeating units of one to several monosaccharides/monomers.

[0145] CP5 refers to *Staphylococcus aureus* type 5 capsular polysaccharide or serotype 5 capsular polysaccharide.

[0146] CP8 refers to *Staphylococcus aureus* type 8 capsular polysaccharide or serotype 8 capsular polysaccharide.

[0147] D-FucNAc refers to N-acetyl D-fucosamine.

[0148] ECA refers to enterobacterial common antigen.

[0149] ELISA refers to Enzyme-linked immunosorbent assay, a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.

[0150] EPA or EPAr refers to nontoxic recombinant *P. aeruginosa* exoprotein A.

[0151] Glycoconjugate vaccine refers to a vaccine comprising a protein carrier linked to an antigenic or immunogenic oligosaccharide.

[0152] Glycosyltransferase refers to enzymes that act as a catalyst for the transfer of a monosaccharide unit from an activated nucleotide sugar to a glycosyl acceptor molecule.

[0153] Gram-positive strain refers to a bacterial strain that stains purple with Gram staining (a valuable diagnostic tool). Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (approximately 50-90% of cell wall).

[0154] Gram-negative strain refers to a bacterial strain which has a thinner layer (approximately 10% of cell wall) which stains pink. Gram-negative bacteria also have an additional outer membrane that contains lipids, and is separated from the cell wall by the periplasmic space.

[0155] Hla (alpha toxin) refers to alpha hemolysin, which is a secreted pore-forming toxin and an essential virulence factor antigen of *S. aureus*.

[0156] Hla H35L refers to a mutant form of Hla nontoxic alpha-toxin mutant from *S. aureus*.

[0157] Histidine tag, or polyhistidine-tag, is an amino acid motif in proteins that consists of at least five histidine (His) residues, often at the N- or C-terminus of the protein, and used to purify in a simple and fast manner by specifically binding to a nickel affinity column.

[0158] IV refers to intravenously.

[0159] kDa refers to kilo Daltons, is an atomic mass unit.

[0160] L-FucNAc refers to N-acetyl L-fucosamine.

[0161] LPS refers to lipopolysaccharide. Lipopolysaccharides (LPS), also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals.

[0162] ManNAcA refers to N-acetyl mannosaminuronic acid.

[0163] Methicillin-resistant *S. aureus* strains (MRSA) refers to methicillin-resistant *S. aureus* strain associated with longer hospital stay and more infections in intensive care units which leads to more antibiotic administration.

[0164] *N*-glycans or *N*-linked oligosaccharides refers to mono-, oligo- or polysaccharides of variable compositions that are linked to an ε-amide nitrogen of an asparagine residue in a protein via an *N*-glycosidic linkage.

[0165] *N*-linked protein glycosylation refers to a process or pathway to covalently link "glycans" (mono-, oligo- or polysaccharides) to a nitrogen of asparagine (N) side-chain on a target protein.

[0166] O-antigens or O-polysaccharides refers to a repetitive glycan polymer contained within an LPS. The O antigen is attached to the core oligosaccharide, and comprises the outermost domain of the LPS molecule.

[0167] Oligosaccharides or Polysaccharides refers to homo- or heteropolymer formed by covalently bound carbohydrates (monosaccharides), and includes but is not limited to repeating units (monosaccharides, disaccharides, trisaccharides, etc.) linked together by glycosidic bonds.

[0168] Opsonophagocytic activity refers to phagocytosis of a pathogen in the presence of complement and specific antibodies. The in vitro opsonophagocytic activities (OPAs) of serum antibodies are believed to represent the functional activities of the antibodies in vivo and thus to correlate with protective immunity.

[0169] OTase or OST refers to oligosaccharyl transferase, which catalyzes a mechanistically unique and selective transfer of an oligo- or polysaccharide (glycosylation) to the asparagine (N) residue at the consensus sequence of nascent or folded proteins.

[0170] Passive immunization is the transfer of active humoral immunity in the form of already made antibodies, from one individual to another.

[0171] Periplasmic space refers to the space between the inner cytoplasmic membrane and external outer membrane of Gram-negative bacteria.

[0172] PMNs refers to polymorphonuclear neutrophils, which are the most abundant white blood cells in the peripheral blood of humans, and many (though not all) mammals.

[0173] Protein carrier refers to a protein that comprises the consensus sequence into which the oligo- or polysaccharide is attached.

[0174] RU refers to a repeating unit comprising specific polysaccharides synthesized by assembling individual monosaccharides into an oligo- or polysaccharide.

[0175] Signal sequence refers to a short (e.g., approximately 3-60 amino acids long) peptide at the N-terminal end of the protein that directs the protein to different locations.

[0176] UDP-D-ManNAc is UDP-N-acetyl-D-mannosamine.

[0177] UDP-D-ManNAcA is UDP-N-acetyl-D-mannosaminuronic acid.

[0178] UDP-D-QuiNAc is UDP-N-acetyl-D-quinovosamine.

[0179] UDP-L-FucNAc is UDP-N-acetyl-L-fucosamine.

[0180] UDP-L-6dTalNAc is UDPN-acetyl-L-pneumosamine.

[0181] Und refers to undecaprenyl or undecaprenol lipid composed by eleven prenol units.

[0182] UndP refers to undecaprenyl phosphate, which is a universal lipid carrier (derived from Und) of glycan biosynthetic intermediates for carbohydrate polymers that are exported to the bacterial cell envelope.

[0183] UndPP refers to undecaprenyl pyrophosphate, which is a phosphorylated version of UndP.

[0184] wbjA is a glucosyltransferase in *P. aeruginosa* O11.

[0185] wbjB is a putative epimerase similar to enzymes required to the capsule biosynthesis of CP5 and CP8 in *S. aureus*.

[0186] wbjC is a putative epimerase in *P. aeruginosa* O11.

[0187] wbjD is a putative epimerase in *P. aeruginosa* O11.

[0188] wbjE is a putative epimerase in *P. aeruginosa* O11.

[0189] wbjF is a glycosyltranseferase in *P. aeruginosa* O11.

[0190] wbpL is a glycosyltransferase that participates in LPS biosynthesis in *P. aeruginosa* O11.

[0191] wbpM is a glycosyltransferse that participates in LPS biosynthesis in *P. aeruginosa* O11.

[0192] Embodiments of the invention are at least partially based on the discovery that *C. jejuni* contains a general *N*-linked protein glycosylation system, an unusual feature for prokaryotic organisms. Various proteins of *C. jejuni* have been shown to be modified by a heptasaccharide. This heptasaccharide is assembled on UndPP, the carrier lipid, at the cytoplasmic side of the inner membrane by the stepwise addition of nucleotide activated monosaccharides catalyzed by specific glycosyltransferases. The lipid-linked oligosaccharide is then flipped into (i.e., it diffuses transversely) the periplasmic space by a flippase, e.g., PglK. In the final step of *N*-linked protein glycosylation, the OTase (e.g., PglB) catalyzes the transfer of the oligosaccharide from the carrier lipid to Asn residues within the consensus sequence Asp/Glu-Xaa-Asn-Zaa-Ser/Thr (i.e., D/E – X – N – Z – S/T), where the Xaa and Zaa can be any amino acid except Pro. We had successfully transferred the glycosylation cluster for the heptasaccharide into *E. coli* and were able to produce *N*-linked glycoproteins of *Campylobacter*.

[0193] A novel and inventive method to modify a Gram-negative host bacterium, such as *E. coli*, has been developed to produce glycosylated proteins for use as vaccine products against a Gram-positive bacterium such as *S. aureus*. The development of this method required overcoming significant and in many respects unexpected problems, and departing substantially from conventional wisdom and the prior art.

[0194] In this novel and inventive method, another Gram-negative bacterium was identified that produces a polysaccharide that has structural similarity to the polysaccharide of interest of the target organism, for example *S. aureus*. For purposes of this invention, structural similarity manifests itself as repeating units in the polysaccharide of the target (e.g., *S. aureus*) that are partially identical to repeating units in the polysaccharide of the identified, other Gram-negative bacterium. Because this latter bacterium is Gram-negative, as is the host, for example, *E. coli* organism, we initially hypothesized (and later verified by experiment as discussed below) that use of its biosynthesis pathways in a modified *E. coli* organism would allow the biosynthesis of the constructed RU antigen and its flipping from the cytoplasm into the periplasm of the modified *E. coli* organism. Further, we hypothesized (and later verified by experiment as discussed below) that the size of the polysaccharide produced through this biosynthesis pathway would be much smaller than the polysaccharide produced by the biosynthesis pathway of Gram positive *S. aureus*.

[0195] As a result, and as discussed below, the novel and innovative method we developed solved the aforementioned difficult problems.

[0196] Furthermore, it was surprisingly found that aspects of the LPS pathway in a Gram-negative organism could be used to produce polysaccharides that contain some of the same repeating units as capsular polysaccharides native to Gram-positive bacteria, such as, for example, *S. aureus*, as detailed below.

[0197] Therefore, in making the polysaccharide section of the glycosylated protein vaccine for *S. aureus*, one surprising solution is to construct the polysaccharide section at least partially based on a polysaccharide native to a Gram-negative bacterium like *E. coli*. We further discovered that, in doing so, it is apparently important to find a bacterium which produces a polysaccharide that is as similar as possible to the polysaccharide of interest produced by *S. aureus*. *P. aeruginosa* is such a bacterium.

[0198] FIG. 1 provides a step-by-step depiction of an embodiment of the preparation of nucleotide-activated monosaccharides in the cytoplasm either by enzymes provided in the O-antigen cluster or by house keeping enzymes of the Gram-negative host cell, as would be apparent to one of skill in the art in light of this specification. The steps of the process proceed from left to right in the depiction of FIG. 1. In the embodiment depicted in FIG. 1, a glycosylphosphate transferase (WbpL) adds D-FucNAc phosphate to UndP, forming UndPP-FucNAc. Specific glycosyltransferases then elongate the UndPP-D-FucNAc molecule further by adding monosaccharides forming the repeating unit (RU) oligosaccharide (WbjE, WbjA). The RU is then flipped into the periplasmic space by the Wzx protein. The Wzy enzyme polymerizes periplasmic RUs to form the O-antigen polysaccharide. Polymer length is controlled by the Wzz protein. Many bacterial oligo- and polysaccharides are assembled on UndPP and then transferred to other molecules. In other words, UndPP is a general building platform for sugars in bacteria. In *E. coli* and, it is believed, most other Gram negative bacteria, the O-antigen is transferred from UndPP to Lipid A core by the *E. coli* enzyme WaaL to form lipopolysaccharide (LPS).

[0199] FIG. 2 depicts an embodiment of preparation of nucleotide-activated monosaccharides in the cytoplasm by enzymes provided in the O-antigen cluster of *P. aeruginosa* O11, by house keeping enzymes of the Gram-negative host cell, and by *S. aureus* and/or *E. coli* enzymes known to be required for UDP-ManNAcA biosynthesis (Cap5OP and/or WecBC), as would be apparent to one of skill in the art in light of this specification. In the depiction of FIG. 2, the steps of the process proceed from left to right. As in O11 biosynthesis, WbpL and WbjE synthesize the core disaccharide. Then, the *S. aureus* glycosyltransferase Cap5I adds D-ManNAcA. Cap5H adds an acetyl group to the second FucNAc residue. Acetylation may be the final step of RU synthesis as shown in FIG. 2. Flipping is possible by one or all of the Wzx proteins in the system, which are recombinantly expressed Wzx of *P. aeruginosa* or Cap5K, or endogenously expressed Wzx-like enzymes e.g. of the ECA cluster encoded in the *E. coli* chromosome. Polymerization is an exclusive activity of the Cap5J polymerase forming the CP5 polysaccharide on UndPP. As other UndPP linked polysaccharides, the CP5 sugar is transferred to Lipid A core by the *E. coli* enzyme WaaL to form recombinant LPS (LPS capsule).

[0200] FIG. 3 depicts the preparation of nucleotide-activated monosaccharides in the cytoplasm by enzymes provided in the O-antigen cluster of *P. aeruginosa* O11, by house keeping enzymes of the Gram-negative host cell, and by *S. aureus* and/or *E. coli* enzymes known to be required for UDP-ManNAcA biosynthesis (Cap8OP and/or WecBC), as would be apparent to one of ordinary skill in the art in light of this specification. In the depiction of FIG. 3, the steps of the process proceed from left to right. As in O11 biosynthesis, WbpL and WbjE synthesize the core disaccharide. Then, the *S. aureus* glycosyltransferase Cap8H adds D-ManNAcA. Cap8J adds an acetyl group to the second FucNAc residue. It is not known if acetylation occurs on the activated sugar or the lipid bound RU. Flipping is possible by one or all of the Wzx proteins in the system, which are recombinantly expressed Wzx of *P. aeruginosa* or Cap8K, or endogenously expressed Wzx-like enzymes e.g. of the ECA cluster encoded in the *E. coli* chromosome. Polymerization is an exclusive activity of the Cap8I polymerase forming CP8 polysaccharide on UndPP. The CP8 sugar is then transferred to Lipid A core in *E. coli* by the enzyme WaaL.

[0201] FIG. 4 illustrates the different structures of the O11, CP5 and CP8 polysaccharides. It is shown in FIG. 4 that the RUs share the identical stem structure consisting of the UndPP and the disaccharide α -D-FucNAc-(1,3)-L-FucNAc. The *S. aureus* RUs are partially decorated with a single O-acetyl group, either on the middle L-FucNAc or on the ManNAcA residue, which is characteristic for the *S. aureus* RUs. The connectivity of the second and third sugar in the *S. aureus* RUs is different between them, as well as the connectivity between the polymerized RUs. On the right, the sugar structures are shown in a different representation. The number by the back arrows (CP5 and CP8) indicates the position of the carbon modified with an O-acetyl group. An alternative representation of the RU structures is shown on the bottom left. As shown in FIG. 4, there is great overlap between the RU in the O11 antigen that is part of a polysaccharide native to *P. aeruginosa* and those of the CP5 and CP8 capsules of the respective strains of *Staphylococcus*. In particular, as show in FIG. 4, the L-FucNAc-->D-FucNAc portion in the RU it is identical in both.

[0202] In another aspect, the invention features a method of identifying a target polysaccharide for use in glycosylating a protein with said target polysaccharide, in whole or in part. Said glycosylated protein comprising the target polysaccharide can be used, for example, in vaccine compositions. The method of identifying a target polysaccharide includes: identifying a Gram-positive bacterium, such as *S. aureus*, as a target; identifying a first repeating unit of a polysaccharide produced by said Gram-positive bacterium comprising at least three monomers; identifying a polysaccharide produced by a bacterium of a Gram-negative species comprising a second repeating unit comprising at least two of the same monomers as said first repeating monomer unit.

[0203] Accordingly, in one embodiment of the invention, a method of modifying a bacterium of a first Gram-negative species includes: identifying a Gram-positive bacterium, such as *S. aureus*, as a target; identifying a first repeating unit of a polysaccharide produced by said Gram-positive bacterium comprising at least three monomers; identifying a polysaccharide produced by a bacterium of a second Gram-negative species comprising a second repeating unit comprising at least two of the same monomers as said first repeating unit; inserting into said bacterium of a first Gram-negative species one or more nucleotide sequences encoding glycosyltransferases that assemble a trisaccharide containing: a) said second repeating unit; and b) a monomer of said first repeating unit not present in said second repeating unit; inserting a nucleotide sequence encoding a protein, such as a protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and inserting a nucleotide sequence encoding an OTase.

[0204] In an embodiment of the invention, the method further comprises inserting into a host Gram-negative bacterium one or more nucleotide sequences encoding glycosyltransferases that assemble a trisaccharide containing a monomer of a first repeating unit not present in a second repeating unit and that assemble the second repeating unit. An additional embodiment of the invention involves inserting one or more glycosyltransferases from a Gram-negative bacterium that assemble at least one monomer unit from a first repeating unit and one or more glycosyltransferases from a Gram-positive bacterium, such as *S. aureus*, that assemble at least two monomers from a second repeating unit. The method additionally comprises inserting into inserting into a Gram-negative host bacterium a nucleotide sequence encoding a protein and a nucleotide sequence encoding an OTase.

[0205] In at least one embodiment of the invention, a host *E. coli* strain is generated carrying the corresponding nucleic acids encoding the required enzymes from the CP5 and CP8 strains of *S. aureus*, which will build up, flip and polymerize the constructed repeating units. In an embodiment, the specific glycosyltransferases needed correspond to those forming the L-FucNAc-->D-FucNAc RU that are native to *P. aeruginosa*, and to glycosyltransferases corresponding to the ones adding the D-ManNAcA monosaccharide to the complete the RU that are native to each of the CP5 and CP8 strains of *S. aureus*. Such an embodiment may further include using a plasmid to inject the nucleic acids into the host cell. An additional embodiment involves using, in one plasmid, nucleic acids encoding for the glycosyltransferases corresponding to L-FucNAc-->D-FucNAc, and, in a different plasmid, nucleic acids encoding for the glycosyltransferases corresponding to D-ManNAcA. One benefit of such embodiments, surprising in light of the prior art, is that the modified LPS biosynthesis pathway of *P. aeruginosa* that is now responsible for producing the constructed RU polymer of the *S. aureus* capsule results in a structure that is much smaller than the capsule of *S. aureus*.

[0206] The instant invention is additionally directed to a recombinant *N*-glycosylated protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and at least one oligo- or polysaccharide from a Gram-positive bacterium linked to said consensus sequence. In another embodiment, the recombinant *N*-glycosylated protein comprises two or more of said inserted consensus sequences. In yet an additional embodiment, the recombinant *N*-glycosylated protein comprises two or more of said *S. aureus* oligo- or polysaccharides. In a still further embodiment, the recombinant *N*-glycosylated protein comprises two or more of said inserted consensus sequences and oligo- or polysaccharides from different *S. aureus* strains, for example, from *S. aureus* capsular polysaccharide 5 strain and capsular polysaccharide 8 strain.

[0207] The present invention is furthermore directed to a combination of a modified capsular polysaccharide of *S. aureus* with a protein antigen from the same organism by *N*-glycosidic linkage.

[0208] Embodiments of the present invention include a protein that is glycosylated in nature. Such naturally glycosylated proteins (e.g., *C. jejuni* proteins) contain natural consensus sequences but do not comprise any additional (*i.e.*, introduced) optimized consensus sequences. Naturally glycosylated proteins include prokaryotic and eukaryotic proteins. Embodiments of the instant invention further include a recombinant N-glycosylated protein, comprising one or more of the following N-glycosylated partial amino acid sequence(s): D/E - X - N - Z - S/T, (optimized consensus sequence) wherein X and Z may be any natural amino acid except Pro, and wherein at least one of said N-glycosylated partial amino acid sequence(s) is introduced. The introduction of specific partial amino acid sequence(s) (optimized consensus sequence(s)) into proteins leads to proteins that are efficiently *N*- glycosylated by an OTase, such as, for example, an OTase from *Campylobacter* spp., such as, for example, an OTase from *C. jejuni*, at the positions of introduction.

[0209] The term "partial amino acid sequence(s)" as it is used in the context of the present invention will also be referred to as "optimized consensus sequence(s)" or "consensus sequence(s)". The optimized consensus sequence is *N*-glycosylated by an OTase, such as, for example, an OTase from *Campylobacter* spp., such as, for example, an OTase from *C. jejuni*.

[0210] In accordance with the internationally accepted one letter code for amino acids the abbreviations D, E, N, S and T denote aspartic acid, glutamic acid, asparagine, serine, and threonine, respectively.

[0211] The introduction of the optimized consensus sequence can be accomplished by the addition, deletion and/or substitution of one or more amino acids. The addition, deletion and/or substitution of one or more amino acids for the purpose of introducing the optimized consensus sequence can be accomplished by chemical synthetic strategies well known to those skilled in the art such as solid phase-assisted chemical peptide synthesis. Alternatively, and preferred for larger polypeptides, the proteins of the present invention can be prepared by standard recombinant techniques by adding nucleic acids encoding for one or more optimized consensus sequences into the nucleic acid sequence of a starting protein, which may be a protein that is naturally glycosylated or may be a protein that is not naturally glycosylated.

[0212] In a preferred embodiment, the proteins of the present invention may comprise one or more, preferably at least two or at least three, and more preferably at least five of said introduced *N*-glycosylated optimized amino acid sequences.

[0213] The presence of one or more *N*-glycosylated optimized amino acid sequence(s) in the proteins of the present invention can be of advantage for increasing their antigenicity, increasing their stability, affecting their biological activity, prolonging their biological half-life and/or simplifying their purification.

[0214] The optimized consensus sequence may include any amino acid except proline in position(s) X and Z. The term "any amino acids" is meant to encompass common and rare natural amino acids as well as synthetic amino acid derivatives and analogs that will still allow the optimized consensus sequence to be N-glycosylated by the OTase. Naturally occurring common and rare amino acids are preferred for X and Z. X and Z may be the same or different.

[0215] It is noted that X and Z may differ for each optimized consensus sequence in a protein according to the present invention.

[0216] The *N*-glycan bound to the optimized consensus sequence will be determined by the specific glycosyltransferases and their interaction when assembling the oligosaccharide on a lipid carrier for transfer by the OTase. Those skilled in the art can design the *N*-glycan by varying the type(s) and amount of the specific glycosyltransferases present in the desired host cell. (Raetz & Whitfield, Lipopolysaccharide Endotoxins, *NIH-PA Author Manuscript* 1-57, 19-25 (published in final edited form as: *Annual Rev. Biochem.*, 71: 635-700 (2002)); Reeves *et al.*, Bacterial Polysaccharide Synthesis and Gene Nomenclature, Trends in Microbio. 4(3): 495-503, 497-98 (Dec.

1996); and Whitfield, C. and I. S. Roberts. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli.* Mol Microbiol 31(5): 1307-19).

[0217] "Polysaccharides" as used herein include saccharides comprising at least two monosaccharides. Polysaccharides include oligosaccharides, trisaccharides, repeating units comprising one or more monosaccharides (or monomers), and other saccharides recognized as polysaccharides by one of ordinary skill in the art. *N*-glycans are defined herein as mono-, oligo- or polysaccharides of variable compositions that are linked to an ε-amide nitrogen of an asparagine residue in a protein via an *N*-glycosidic linkage.

[0218] Polysaccharides of embodiments of the invention include without limitation *S. aureus* polysaccharides such as CP5 and CP8. Embodiment of the invention further includes *S. aureus* polysaccharides that target a bacterium, such as a polysaccharide that targets a methicillin-resistant strain of *S. aureus*. Where it is mentioned herein that polysaccharides target a bacterial strain, such polysaccharides include polysaccharides that are from the bacterium against which an immune or antigenic response is desired and further include polysaccharides that are the same as, based on, derived from, native to or engineered from the bacterium against which an immune or antigenic response is desired.

[0219] There is no limitation on the origin of the recombinant protein of the invention. In one embodiment, said protein is derived from mammalian, bacterial, viral, fungal or plant proteins. In a further embodiment, the protein is derived from mammalian, most preferably human proteins. For preparing antigenic recombinant proteins according to the invention, preferably for use as active components in vaccines, it is preferred that the recombinant protein is derived from a bacterial, viral or fungal protein. Glycosylation of proteins of various origins is known to one of skill in the art. Kowarik *et al.* "Definition of the bacterial *N*-glycosylation site consensus sequence" EMBO J. (2006) 1-10.

[0220] In an example in an embodiment, genetically detoxified *P. aeruginosa* Exotoxin (EPA) is a suitable protein carrier. For producing a version of EPA that may be glycosylated, the nucliec acids encoding for EPA need to be modified by insertion of glycosylation sites as previously discussed.

[0221] Protein carriers intended for use in embodiments of the invention should preferably have certain immunological and pharmacological features. From an immunological perspective, preferably, a protein carrier should: (1) have T-cell epitopes; (2) be capable of delivering an antigen to antigen presenting cells (APCs) in the immune system; (3) be potent and durable; and (4) be capable of generating an antigen-specific systemic IgG response. From a pharmacological perspective, a protein carrier should preferably: (1) be non-toxic; and (2) be capable of delivering antigens efficiently across intact epithelial barriers. More preferably, in addition to these immunological and pharmacological features, a protein carrier considered for use in the production of a bacterial bioconjugate should: (1) be easily secreted into the periplasmic space; and (2) be capable of having antigen epitopes readily introduced as loops or linear sequences into it. Informed by this disclosure and knowledge of one of ordinary skill in the art, a practitioner of ordinary skill in the art may routinely consider and identify suitable protein carriers that may be used in particular embodiments of the invention.

[0222] In an embodiment of the invention, the *Campylobacter* protein AcrA is a protein carrier.

[0223] In a further embodiment of the invention, genetically detoxified *P. aeruginosa* Exotoxin (EPA) is a protein carrier in which the target organism for which a vaccine is desired is *S. aureus*. Unlike AcrA which contains natural glycosylation sites, EPA contains no such natural glycosylation sites and needs to be modified by insertion of glycosylation sites (e.g., insertion of nucleic acids encoding for the optimized consensus sequence as discussed earlier into the nucleic acid sequence encoding for EPA). In an additional embodiment, EPA is modifed to introduce two glycosylation sites that will allow glycosylation with the *S. aureus* antigen. In a still further embodiment, two consensus sequences are introduced as discussed in Example 10 of WO 2009/104074.

[0224] The amino acid sequence of EPA, as modified in an embodiment of this invention to contain two glycosylation sites, is provided as SEQ ID NO: 13 (with signal sequence) and SEQ ID NO.: 14 (without signal sequence). The glycosylation sites in SEQ ID NO: 13 are

DNNNS and DQNRT at positions 260DNNNS and 402DQNRT. The glycosylation sites in SEQ ID NO: 14 are DNNNS and DQNRT at positions 241DNNNS and 383DQNRT.

[0225] A carrier protein such as EPA is a protein on which N-glycosylation sites may be added in the production of a bacterial bioconjugate. N-glycosylation sites require introduction of the consensus sequences discussed previously, namely, insertion of D/E – X – N – Z-S/T sequons, wherein X and Z may be any natural amino acid except proline. We have found that such consensus sequences preferably are introduced in surface loops, by insertion rather than mutation and by the use of additionally inserted flanking residues and by mutation of flanking residues to optimize the operation of the N-glycosylation site.

[0226] Some well-characterized protein subunit antigens of *S. aureus* are the alpha hemolysin (alpha toxin, Hla), clumping factor alpha (ClfA), IsdB, and Panton-Valentine Leukocidin (PVL).

[0227] Hla is a secreted pore-forming toxin and an essential virulence factor of MRSA in a mouse model of *S. aureus* pneumonia. The level of Hla expression by independent *S. aureus* strains directly correlates with their virulence. Active immunization with a mutant form of Hla (Hla H35L, SEQ ID NO: 5), which cannot form pores (Menzies, B. E., and D. S. Kernodle. 1996. Passive immunization with antiserum to a nontoxic alpha-toxin mutant from *Staphylococcus aureus* is protective in a murine model. Infect Immun 64:1839-41; Jursch, R., A. Hildebrand, G. Hobom, J. Tranum-Jensen, R. Ward, M. Kehoe and S. Bhakdi. 1994. Histidine residues near the N terminus of staphylococcal alpha-toxin as reporters of regions that are critical for oligomerization and pore formation. Infect Immun 62(6): 2249-56), was shown to generate antigen-specific immunoglobulin G responses and to afford protection against staphylococcal pneumonia. Transfer of Hla-specific antibodies protects naive animals against *S. aureus* challenge and prevents the injury of human lung epithelial cells during infection (Bubeck Wardenburg, J., A. M. Palazzolo-Ballance, M. Otto, O. Schneewind, and F. R. DeLeo. 2008. Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. J Infect Dis 198:1166-70). To be used as a vaccine, the H35L mutation in Hla is required to eliminate toxicity of the protein (Menzies, B. E., and D. S. Kernodle. 1994. Site-directed mutagenesis of the alpha-toxin gene of *Staphylococcus aureus*: role of histidines in toxin activity in vitro and in a murine model. Infect Immun 62:1843-7). ClfA contains a protease resistant domain which is used for immunization. Passive immunization of mice with anti-ClfA and anti CP5 antibodies effectively sterilized mammary glands in mammary gland infection model (Tuchscherr, L. P., F. R. Buzzola, L. P. Alvarez, J. C. Lee, and D. O.Sordelli. 2008. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. Infect Immun 76: 5738-44).

[0228] A further embodiment of the invention includes glycosylation of proteins native to *S. aureus*, for example, Hla and ClfA. In additional example embodiments of the invention, the protein carrier used may be selected to be the Hla protein, for example Hla H35L (for example, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 or SEQ ID NO: 16). In another additional example embodiment of the invention, the protein carrier is the ClfA protein (for example, SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12).

[0229] The invention is further directed to recombinant host prokaryotic organisms comprising: a nucleotide sequence encoding one or more glycosyltransferase of a first prokaryotic species, such as a Gram-positive species; one or more glycosyltransferases of a different prokaryotic species, such as a Gram-negative species; a nucleotide sequence encoding a protein; and a nucleotide sequence encoding an OTase. The invention is additionally directed to a recombinant host prokaryotic organism comprising an introduced nucleotide sequence encoding glycosyltransferases native only to a Gram-positive prokaryotic organism; a nucleotide sequence encoding a protein; and a nucleotide sequence encoding an OTase. The invention is also directed to a recombinant or engineered host prokaryotic organism comprising: a nucleotide sequence encoding a glycosyltransferase native to a first prokaryotic species, which is, for example, different from the host prokaryotic organism; a nucleotide sequence encoding a glycosyltransferase native to a second prokaryotic species different from the species of said first prokaryotic organism and, for example, different from said host. The engineered prokaryotic organism can also, for example, comprise a first prokaryotic species that is a Gram-positive species. The engineered prokaryotic organism can also, for example, comprise a second prokaryotic species that is a Gram-negative species. The invention further includes a recombinant or engineered Gram-negative host prokaryotic organism comprising: a nucleotide sequence encoding a glycosyltransferase native to a Gram-negative prokaryotic species that is, for example, different from said host prokaryotic organism; a nucleotide sequence encoding a glycosyltransferase native to *S. aureus*; a nucleotide sequence encoding a protein; and a nucleotide sequence encoding an OTase. The invention further includes a recombinant or engineered *E. coli* host comprising: a nucleotide sequence encoding a glycosyltransferase native to *P. aeruginosa*; a nucleotide sequence encoding one or more glycosyltransferases native to *S. aureus* CP5 strain and/or to *S. aureus* CP8 strain; a nucleotide sequence encoding a *P. aeruginosa* EPA, *S. aureus* alpha hemolysin, or *S. aureus* clumping factor A protein carrier; and a nucleotide sequence encoding an OTase, for example, and OTase native to *C. jejuni*.

[0230] In addition to using the biosynthesis pathway of the other Gram-negative organism in the modified host *E. coli* organism, in a further embodiment, also included within the host *E. coli* organism are nucleic acids encoding for (i) glycosyltransferases for construction the structure of the repeating units of the polysaccharide of the other Gram-negative organism (that are identical to the repeating units of the polysaccharide of interest of the target Gram-positive *S. aureus* organism), and (ii) glycosyltransferases for construction of the units of the polysaccharide of interest of the target Gram-positive *S. aureus* organism that are not found in the relevant polysaccharide of the other Gram-negative organism, and (iii) enzymes for flipping and polymerization of the constructed RU of interest of the target Gram-positive *S. aureus* organism to form a *S. aureus* capsule like polysaccharide. In particular, in this embodiment, the nucleic acids encoding for (i) originated with the other Gram-negative bacterium, whereas the nucleic acids encoding for (ii) and (iii) originated with the target Gram-positive *S. aureus* organism.

[0231] Another aspect of the invention is directed to: an engineered host prokaryotic organism comprising: i) a nucleotide sequence encoding glycosyltransferases native to a Gram-positive prokaryotic species; ii) a nucleotide sequence encoding a protein; and iii) a nucleotide sequence encoding an OTase, wherein the sequences encoding transporter genes of said Gram-positive prokaryotic species are deleted. Such an embodiment involves an introduced nucleic acid construct that encodes only Gram-positive glycosyltransferases.

[0232] Regarding the other nucleic acids that would be inserted into the host in one or more other embodiments, nucleic acids encoding a protein, such as AcrA, Hla, ClfA or EPA (SEQ ID NO: 15, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 16; SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12; SEQ ID NO: 13, SEQ ID NO: 14), and the oligosaccharyltransferase of *C. jejuni* (SEQ ID NO: 27), which are part of the glycosylation machinery of that organism, are injected into the host in addition to the nucleic acids encoding for glycosyltransferases from each of *P. aeruginosa* and *S. aureus*. As a result, the modified *E. coli* organism can glycosylate the AcrA protein with the polysaccharide produced in that organism by action of the glycosyltransferases from *S. aureus* and the other Gram-negative bacterium.

[0233] One embodiment of the invention involves an engineered host prokaryotic organism comprising: i) a nucleotide sequence encoding a glycosyltransferase native to a first prokaryotic species different from the host prokaryotic organism; ii) a nucleotide sequence encoding a glycosyltransferase native to a second prokaryotic species, for example, a Gram-positive prokaryotic species, different from the host prokaryotic organism; iii) a nucleotide sequence encoding a protein; and iv) a nucleotide sequence encoding an OTase. In embodiments of the invention, the first prokaryotic species is a Gram-negative species, for example, *P. aeruginosa.*

[0234] In the context of the present invention, host cells refer to any host cell, e.g., an eukaryotic or prokaryotic host cell. In other embodiments the host cell is a prokaryotic host cell, e.g. *Escherichia* ssp., *Campylobacter* ssp., *Salmonella* ssp., *Shigella* ssp., *Helicobacter* ssp., *Pseudomonas* ssp. or *Bacillus* ssp. In still further embodiments, the host cell is *Escherichia coli, Campylobacter jejuni, Salmonella typhimurium*, etc.

[0235] The invention is furthermore directed to methods of producing a bioconjugate vaccine comprising introducing into a host prokaryotic organism nucleic acids encoding one or more glycosyltransferases of *S. aureus*; one or more glycosyltransferases of a second prokaryotic species, a protein; and an OTase. In addition, the present invention is directed to the production of bioconjugate vaccines by producing in Gram-negative bacteria modified capsular polysaccharides on undecaprenol (Und), and linking these polysaccharide antigens to a protein carrier of choice.

[0236] The invention is further directed to methods of producing glycosylated proteins in a host prokaryotic organism comprising nucleotide sequence encoding glycosyltransferases native to a first prokaryotic organism and also encoding glycosyltransferases native to a second prokaryotic organism that is different from the first prokaryotic organism. The present invention is additionally directed to the production of proteins *N*-glycosylated with capsular polysaccharides of Gram-positive bacteria, which are synthesized by a combination of different glycosyltransferases from different organisms. The invention is furthermore directed to the production of glycosylated proteins in a host prokaryotic organism comprising an introduced nucleotide sequence encoding glycosyltransferases native only to a Gram-positive prokaryotic organism.

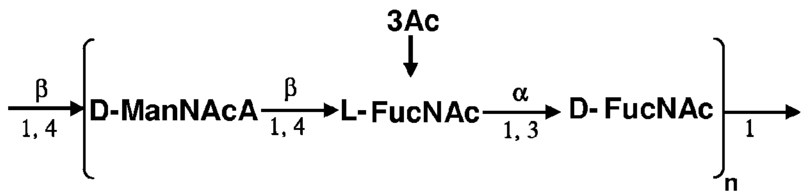
[0237] As in known in the art, the biosynthesis of different polysaccharides is conserved in bacterial cells. The polysaccharides are assembled on carrier lipids from common precursors (activated sugar nucleotides) at the cytoplasmic membrane by different glycosyltransferases with defined specificity. (Whitfield, C., and I. S. Roberts. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol 31: 1307-19). The biosynthetic pathway for polysaccharide production of O-antigen in Gram-negative and for capsular polysaccharide Type I in Gram-positive is conserved. The process uses the same lipid carrier, i.e., UndP, for polysaccharide assembly. It starts with the addition of a monosaccharide-1-phosphate to the carrier lipid UndP at the cytoplasmic side of the membrane. The antigen is built up by sequential addition of monosaccharides from activated sugar nucleotides by different glycosyltransferases. The lipid-linked oligosaccharide or RU is then flipped through the membrane by the flippase. RUs are polymerized by the enzyme Wzy in the periplasmic space, forming the so-called O-antigen in Gram negative bacteria or capsular polysaccharide in Gram-positive bacteria. Gram negative bacteria use the Wzz enzyme to regulate the length of the polymer, which is then transferred to lipid A core forming LPS. LPS is further translocated to the outer membrane exposing the O-antigen to the outside (as depicted, for example, in FIG. 1). Gram-positive bacteria, in contrast, form the capsule from this lipid-bound precursor by further transport using a different and specialized enzymatic machinery. The biosynthetic pathways of these polysaccharides enable the production of bioconjugates *in vivo* by capturing the polysaccharides in the periplasm onto a protein carrier.

[0238] The process of polysaccharide construction differs for capsular polysaccharides in that the capsular polysaccharide is released from the carrier lipid after polymerization and exported to the surface. In Gram-positive bacteria like *S. aureus* that do not contain a periplasmic compartment, the polymerization of the antigen takes place at the outer side of the membrane. In addition, length regulation in *S. aureus* is included in the machinery of three enzymes responsible for capsule assembly. In this assembly, the polysaccharide is released from the carrier lipid and exported to the surface by an enzymatic process.

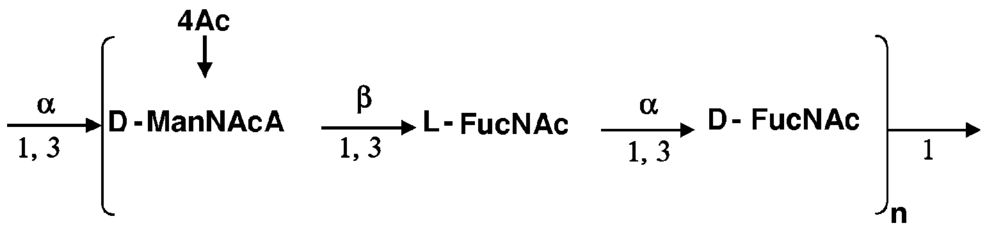
[0239] The genetic elements found in the gene cluster required for functional capsule expression in *S. aureus* resemble the genetic machinery found in wzy dependent O-antigen synthesis clusters. (Dean, C. R., C. V. Franklund, J. D. Retief, M. J. Coyne, Jr., K. Hatano, D. J. Evans, G. B. Pier, and J. B. Goldberg. 1999. Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. J Bacteriol 181:4275-4284).

[0240] Despite these differences between polysaccharide construction in Gram-positive and Gram-negative bacteria, it was surprisingly discovered and verified that aspects of the LPS pathway in a Gram-negative organism could be used to produce polysaccharides that contain some of the same repeating units as capsular polysaccharides native to Gram-positive bacteria, such as, for example, *S. aureus*. As such polysaccharides are produced by LPS pathway mechanisms in the Gram-negative host, the structure of such polysaccharides is the same as in LPS polysaccharide precursors. Such polysaccharides produced in Gram-negative systems of the instant invention can be characterized, therefore, as “modified capsular polysaccharides” or “LPS capsules” for purposes of this application. Furthermore, this newly synthesized expression system and biosynthetic pathway, which combines the LPS and capsular biosynthetic pathways, may be characterized as being a “modified LPS biosynthetic pathway” for purposes of this application.

[0241] In one embodiment of the present invention, a modified polysaccharide produced by a modified LPS biosynthetic pathway comprises:



[0242] In a further embodiment of the present invention, a modified polysaccharide produced by a modified LPS biosynthetic pathway comprises:



[0243] Using the technology of the invention, bacterial bioconjugates can be produced that are immunogenic. Genetic modifications can be made allowing *in vivo* conjugation of bacterial polysaccharides in desired proteins and at desired positions.

[0244] Another aspect of the invention involves production of LPS-capsules or modified LPSs conjugated to a protein carrier using the modified LPS biosynthetic pathway as discussed above.

[0245] A further embodiment of the invention includes a nucleotide sequence construct that encodes the Cap5 and Cap8 complete polysaccharide biosynthesis cluster, wherein the deleted transporter genes are *capA*, *capB* and *capC* of *S. aureus* (see FIG. 6).

[0246] An additional embodiment of the invention includes integrating the CP5/O11 chimeric cluster (SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 17) or the CP8/O11 chimeric cluster (SEQ ID NO. 4, SEQ ID NO. 18 or SEQ ID NO. 19) into the genome of a host cell. A further embodiment of the invention involves integrating into the genome of a host cell: (a) the

CP5/O11 chimeric cluster (SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 17) or CP8/O11 chimeric cluster (SEQ ID NO. 4 SEQ ID NO. 18 or SEQ ID NO. 19); (b) nucleic acids encoding the OTase; and (c) nucleic acids encoding a protein with or without an introduced consensus sequence.

[0247] Another embodiment of the instant invention is directed to plasmids, such as, for example, plasmids comprising one or more of SEQ. ID NO: 2; SEQ. ID NO: 3; SEQ ID NO: 4; SEQ. ID NO: 17; SEQ. ID NO: 18 and SEQ. ID NO: 19. The invention also includes plasmids comprising one or more of SEQ. ID NO: 13; SEQ. ID NO: 14 and SEQ. ID NO: 15. The invention also relates to plasmids comprising one or more of SEQ ID NO: 16; SEQ. ID NO: 6; SEQ. ID NO: 7 and SEQ. ID NO: 8. The invention also relates to plasmids comprising one or more of SEQ ID NO: 10; SEQ. ID NO: 11 and SEQ. ID NO: 12. Moreover, the invention is directed to plasmids comprising one or more of SEQ. ID NO: 20; SEQ. ID NO: 21 and SEQ. ID NO: 27.

[0248] Embodiments of the instant invention furthermore are directed to transformed bacterial cells, such as, for example, including a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO. 2; SEQ. ID NO. 3; SEQ. ID NO: 4; SEQ. ID NO: 17; SEQ. ID NO: 18; SEQ. ID NO: 19; SEQ. ID NO: 20; SEQ. ID NO: 21 and SEQ. ID NO: 27. Further included in the invention is a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO: 19 and SEQ ID NO: 20. Additionally included is a bacterial cell transformed with a plasmid comprising one or more of SEQ ID NO: 13, SEQ ID NO: 19 and SEQ ID NO: 21. The instant invention is further directed to a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO: 16, SEQ ID NO: 6; SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12. The invention is additionally directed to transformed bacterial cells, such as, for example, including a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO. 3; SEQ. ID NO: 4; SEQ. ID NO: 17; SEQ. ID NO: 18; and SEQ. ID NO: 19, and wherein said bacterial cell expresses a glycosyltransferase native to *P. aeruginosa* and a glycosyltransferase native to *S. aureus* CP5 and/or CP8. Further included in the invention is a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO: 17; SEQ ID NO: 18 and SEQ. ID NO: 19 wherein said bacterial cell expresses a glycosyltransferase native to *P. aeruginosa*, a glycosyltransferase native to *S. aureus* CP5 and/or CP8 and PglB. Still further included in the instant invention is (a) a bacterial cell transformed with a plasmid comprising

SEQ. ID NO. 19, wherein said bacterial cell expresses a glycosyltransferase native to *P. aeruginosa*, a glycosyltransferase native to *S. aureus* CP8, Wzz of *E. coli* serovar O7 and PglB; (b) a bacterial cell transformed with a plasmid comprising one or more of SEQ. ID NO. 19 and SEQ. ID NO. 20, wherein said bacterial cell expresses a glycosyltransferase native to *P. aeruginosa*, a glycosyltransferase native to *S. aureus* CP8, Wzz (length regulator), EPA and PglB; and (c) a bacterial cell comprising one or more of SEQ. ID NO. 16; SEQ. ID NO: 6; SEQ. ID NO: 7; SEQ. ID NO: 8; SEQ. ID NO. 13; SEQ. ID NO: 14; SEQ. ID NO: 15; SEQ. ID NO: 10; SEQ. ID NO: 11 and SEQ. ID NO: 12.

[0249] Embodiments of the instant invention are additionally directed to a method of inducing an immune response against an infection caused by Gram-positive and other bacteria in a mammal, such as, for example, in a human. In one embodiment, the method comprises administering to said mammal an effective amount of a pharmaceutical composition comprising: protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and one or more oligo- or polysaccharides, the one or more oligo- or polysaccharides being the same or different as another of the one or more oligo- or polysaccharides, from a Gram-positive bacterium linked to said consensus sequence. A further embodiment of the present invention includes a method of inducing an immune response against an infection caused by *S. aureus* in a mammal, comprising administering to said mammal an effective amount of a pharmaceutical composition comprising: an inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; at least one *S. aureus* oligo- or polysaccharide, such as CP5 polysaccharide; and a pharmaceutically acceptable adjuvant. Another embodiment of the invention is directed to inducing an immune response against an infection caused by *S. aureus* in a mammal, comprising administering to said mammal an effective amount of a pharmaceutical composition comprising: a protein comprising an inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; at least one *S. aureus* CP8 polysaccharide; and a pharmaceutically acceptable adjuvant. A still further embodiment is directed to inducing an immune response against an infection caused by *S. aureus* in a mammal, comprising administering an effective amount of a pharmaceutical composition comprising a protein with two or more consensus sequences and oligo- or polysaccharides from different Gram-positive bacterial strains. A still further embodiment is directed to inducing an immune response against an infection caused by *S. aureus* in a mammal, comprising administering an effective amount of a pharmaceutical composition comprising a protein with two or more consensus sequences and polysaccharides comprising *S. aureus* CP5 and *S. aureus* CP8.

[0250] In instances in this specification where specific nucleotide or amino acid sequences are noted, it will be understood that the present invention encompasses homologous sequences that still embody the same functionality as the noted sequences. In an embodiment of the invention, such sequences are at least 85% homologous. In another embodiment, such sequences are at least 90% homologous. In still further embodiments, such sequences are at least 95% homologous. The determination of percent identity between two nucleotide or amino acid sequences is known to one of skill in the art.

[0251] Nucleic acid sequences described herein, such as those described in the sequence listings accompanying this specification, are examples only, and it will be apparent to one of skill in the art that these sequences can be combined in different ways. Additional embodiments of the invention include variants of nucleic acids. A variant of a nucleic acid (e.g., a codon-optimized nucleic acid) can be substantially identical, that is, at least 70% identical, for example, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% identical, to SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, 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, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 and/or SEQ ID NO: 27. Nucleic acid variants of a sequence that contains SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO. 3, SEQ ID NO: 4, SEQ ID NO: 5 , SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, 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, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, and/or SEQ ID NO: 27. Include nucleic acids with a substitution, variation, modification, replacement, deletion, and/or addition of one or more nucleotides (e.g., 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500 or more nucleotides) from a sequence that contains SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID. NO: 4, SEQ ID NO: 5 , SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, 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, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 and/or SEQ ID NO: 27, or parts thereof.

[0252] Such variants include nucleic acids that encode prokaryotic glycosyltransferases and that i) are expressed in a host cell such as *E. coli* and ii) are substantially identical to SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 17, SEQ ID NO: 18 and/or SEQ ID NO: 19 and/or parts thereof.

[0253] Nucleic acids described herein include recombinant DNA and synthetic (e.g., chemically synthesized) DNA. Nucleic acids can be double-stranded or single-stranded. In the case of single-stranded nucleic acids, the nucleic acid can be a sense strand or antisense strand. Nucleic acids can be synthesized using oligonucleotide analogs or derivatives, as known to one of skill in the art in light of this specification.

[0254] Plasmids that include a nucleic acid described herein can be transformed into host cells for expression. Techniques for transformation are known to those of skill in the art in light of this specification.

[0255] An additional embodiment of the invention involves producing Gram-positive bioconjugate vaccines containing LPS-capsules or modified LPSs conjugated to a protein carrier.

[0256] A further embodiment of the invention involves a novel bioconjugate vaccine. A further embodiment of the invention involves a novel approach for producing such bioconjugate vaccines that uses recombinant bacterial cells that directly produce immunogenic or antigenic bioconjugates. In one embodiment, bioconjugate vaccines can be used to treat or prevent bacterial diseases, such as diarrhea, nosocomial infections and meningitis. In further embodiments, bioconjugate vaccines may have therapeutic and/or prophylactic potential for cancer or other diseases.

[0257] In another embodiment of the present invention synthesized complexes of polysaccharides (*i.e.*, sugar residues) and proteins (*i.e.*, protein carriers) can be used as conjugate vaccines to protect against infections such as *S. aureus* infections. In one embodiment, a bioconjugate vaccine, such as a Gram-positive vaccine, comprises a protein carrier comprising an inserted nucleic acid consensus sequence; at least one oligo- or polysaccharide from a Gram-positive bacterium linked to the consensus sequence, and, optionally, an adjuvant. The present invention is further directed in another embodiment to a Gram-positive bioconjugate vaccine, such as a *S. aureus* vaccine, comprising a protein carrier comprising an inserted nucleic acid consensus sequence; at least one oligo- or polysaccharide from a Gram-positive bacterium, such as capsular polysaccharide or LPS capsule, linked to the consensus sequence, and, optionally, an adjuvant. In another embodiment of the invention, the *S. aureus* bioconjugate vaccine comprises two or more of these inserted consensus sequences. In a further embodiment, the *S. aureus* bioconjugate vaccine comprises two or more of *S. aureus* oligo- or polysaccharides. A still further embodiment comprises two or more of said inserted consensus sequences and oligo- or polysaccharides from different *S. aureus* strains, for example, from *S. aureus* capsular polysaccharide 5 strain (CP5) and capsular polysaccharide 8 strain (CP8).

[0258] An additional embodiment of the present invention involves an *S. aureus* vaccine made by a glycosylation system using a modified LPS pathway, which comprises the production of a modified capsular polysaccharide or LPS-capsule. A further additional embodiment involves an *S. aureus* vaccine made by a glycosylation system using a modified LPS pathway, which comprises the production of a modified capsular polysaccharide from introduced nucleic acids that do not encode glycosyltransferases of a Gram-negative prokaryotic species.

[0259] A further embodiment involves an *S. aureus* vaccine produced by a glycosylation system comprising nucleic acids encoding: i) one or more glycosyltransferases responsible for producing the L-FucNAc-->D-FucNAc of the RU of the O11 antigen native to *P. aeruginosa*; ii) one or more glycosyltransferases responsible for producing the D-ManNAcA containing RU native to either the CP5 or CP8 strains of *S. aureus*; iii) one or more enzymes responsible for flipping and polymerization of the CP5 or CP8 constructed RUs, iv) a recombinant protein containing introduced consensus sequences; and v) oligosaccharyltransferase from *C. jejuni*. In this embodiment, the host organism may be a Gram-negative bacterium, for example, *E. coli*.

[0260] An additional embodiment of the invention involves an *S. aureus* vaccine produced by a glycosylation system comprising nucleic acids encoding: i) glycosyltransferases responsible for producing the L-FucNAc-->D-FucNAc of the RU of the O11 antigen native to *P. aeruginosa*; ii) a glycosyltransferase responsible for producing the D-ManNAcA containing RU native to either the CP5 or CP8 strains of *S. aureus*; iii) AcrA protein of *C. jejuni*; and iv) oligosaccharyltransferase from *C. jejuni*. In this embodiment, the host organism may be a Gram-negative bacterium, for example, *E. coli*.

[0261] The vaccines of the instant invention have therapeutic and prophylactic utilities. It will be appreciated that the vaccine of the invention may be useful in the fields of human medicine and veterinary medicine. Thus, the subject to be immunized may be a human or other animal, for example, farm animals including cows, sheep, pigs, horses, goats and poultry (e.g., chickens, turkeys, ducks and geese) and companion animals such as dogs and cats.

[0262] In another aspect, the invention is directed to a method of generating vaccines for immunizing a mammal against a bacterium such as a Gram-positive bacterium. The method includes: immunizing a subject with a bioconjugate, such as a bioconjugate comprising a Gram-positive polysaccharide, e.g., an *S. aureus* polysaccharide, and a pharmaceutically acceptable carrier.

[0263] This invention also features vaccine compositions for protection against infection by a gram-positive bacterium such as *S. aureus* or for treatment of gram-positive infection such as *S. aureus* infection. In one embodiment, the vaccine compositions comprise one or more immunogenic components such as a polysaccharide, or a fragment or portion thereof, from *S. aureus*. In a further embodiment, the vaccine compositions comprise one or more immunogenic components such as a protein, or a fragment or portion thereof, from a Gram-negative or Gram-positive bacterium.

[0264] One aspect of the invention provides a vaccine composition for protection against infection by *S. aureus* which contains at least one immunogenic component or fragment of an *S. aureus* polysaccharide and a pharmaceutically acceptable carrier. Such immunogenic components or fragments can include, for example, an *S. aureus* polysaccharide of at least about two monomers in length or at least about three monomers in length. In a further aspect of the invention, an *S. aureus* RU comprises said monomers. Such repeating units can include, for example, an *S. aureus* RU of at least 1 (one) in length.

[0265] Immunogenic components or fragments of the invention can be obtained, for example, by screening polysaccharides or polypeptides produced recombinantly or through chemical synthesis, or, for example, by screening the bioconjugate comprising a polysaccharide and a protein. Screening immunogenic components or fragments of the invention can be performed using one or more of several different assays. For example, screening assays include ELISA and other assays known to one of ordinary skill in the art.

[0266] In one embodiment, immunogenic components or fragments are identified by the ability of the polysaccharide and/or protein to stimulate IgG antibodies against Gram-positive bacteria, such as *S. aureus* CP5 or CP8 polysaccharides, as determined by, for example, the immune response obtained in mice (FIG. 15A) and in rabbit (FIG. 15B) measuring specific anti CP5 antibodies (quantified by ELISA) against the glycoconjugate vaccine candidate CP5-EPA and other means known to a person of ordinary skill in the art.

[0267] In one embodiment, immunogenic components or fragments are identified by the ability of the polysaccharide and/or protein to stimulate opsonic activity, such as opsonophagocytic killing, as determined by, for example by the *S. aureus* killing ("in vitro" activity) with rabbit anti CP5-EPA antibodies (obtained in Example 7 below, see FIG. 15B) and other means known to a person of ordinary skill in the art.

[0268] In yet a further embodiment, immunogenic components or fragments are identified by the ability of the polysaccharide and/or protein to stimulate humoral and/or cell-mediated immunity against Gram-positive bacteria, such as *S. aureus*, as determined by, for example, by protection against bacterial infection ("challenge") using active immunization in mice (FIG. 18) with CP5-EPA and other means known to a person of ordinary skill in the art.

[0269] In an embodiment of the instant invention, a vaccine composition of the invention can be based on a glycoprotein comprising an immunogenic component or fragment of an *S. aureus* polysaccharide of the invention and optionally further comprising a pharmaceutically acceptable carrier or adjuvant. In further embodiments of the instant invention, a vaccine composition can be based on a glycoprotein comprising an immunogenic component or fragment of an *S. aureus* protein of the invention and optionally further comprising a pharmaceutically acceptable carrier or adjuvant. In yet a further aspect of the invention, a vaccine composition can be based on a glycoprotein comprising a immunogenic component or fragment of a *P. aeruginosa* protein of the invention and optionally further comprising a pharmaceutically acceptable carrier and/or adjuvant.

[0270] It is well-known to those of ordinary skill in the art how to modify a vaccine for administration to one mammal type, for example, mice, for administration to another mammal type, for example, humans. For example, one of skill would readily know that deletion of the histidine tag from the protein carrier of a glycoprotein used in a vaccine composition in mice would render the glycoprotein suitable for administration in a vaccine composition in humans. For example, deletion of the HISTIDINE tag (HIS-tag) in protein carriers such as, e.g. EPA (SEQ ID NO: 13), ClfA (SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12), and Hla (SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 16) would be recognized for its use in a glycoprotein for administration to a human.

[0271] It should be understood that amelioration of any of the symptoms of a Gram-positive, for example *S. aureus*, or other bacterial infection or disease is a desirable clinical goal, including a lessening of the dosage of medication used for the Gram-positive-caused infection or disease, for example an *S. aureus*-caused infection or disease, or other bacterial-caused infection or disease, or an increase in the production of antibodies in the serum or mucous of patients. It will be apparent to those skilled in the art that some of the vaccine compositions of the invention are useful for preventing a Gram-positive infection, for example an *S. aureus* infection, or other bacterial infection, some are useful for treating a Gram-positive infection, for example an *S. aureus* infection, or other bacterial infection, and some are useful for both preventing and treating such infections.

[0272] Embodiments of the present invention such as vaccines and other pharmaceutical agents optionally may be prepared using suitable and pharmaceutically acceptable carriers, excipients, diluents and/or adjuvants, as are well-known in the art and apparent in light of this specification. An excipient, diluent or adjuvant may be a solid, semi-solid or liquid material which may serve as a vehicle or medium for the active ingredient. In light of this specification, one of ordinary skill in the art in the field of preparing compositions can readily select the proper form and mode of administration depending upon the particular characteristics of the product selected, the disease or condition to be treated, the stage of the disease or condition, and other relevant circumstances (*Remington's Pharmaceutical Sciences*, Mack Publishing Co. (1990)). The proportion and nature of the pharmaceutically acceptable diluent, excipient or adjuvant are determined by the solubility and chemical properties of the pharmaceutically active compound selected the chosen route of administration and standard pharmaceutical practice.

[0273] Accordingly, in embodiments of the invention, vaccine compositions comprise immunogenic components or fragments, e.g., *S. aureus* polysaccharide or fragment thereof and/or *S. aureus* or *P. aeruginosa* protein or fragment thereof and optionally include a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier that is non-toxic. Suitable pharmaceutically acceptable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody. Such pharmaceutically acceptable carriers include, for example, liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

[0274] Further, in additional embodiments of the invention, the vaccine composition can optionally include an adjuvant or a combination of adjuvants, including, but not limited to particulate adjuvants such as aluminium salts (aluminium hydroxide, aluminium phosphate, aluminium hydroxyphosphate sulphate, etc.); emulsions such as oil in water (MF59, AS03); lipid and salt combinations such as AS04; water in oil (Montanide); ISCOMS, liposomes/virosomes; nano- and microparticles, etc.; non particulated such as peptides; saponins (QS21); MPL A; cytokins; DNA derivates; bacterial toxins; etc. A further embodiment includes adjuvants used in animals such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant, mycolate-based adjuvants (e.g., trehalose dimycolate), bacterial lipopolysaccharide (LPS), peptidoglycans (i.e., mureins, mucopeptides, or glycoproteins such as N-Opaca, muramyl dipeptide [MDP], or MDP analogs), proteoglycans, streptococcal preparations (e.g., OK432), DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), Pluronic, the Ribi adjuvant system or interleukins, particularly those that stimulate cell-mediated immunity. The adjuvant used will depend, in part, on the composition and type of the glycoconjugate vaccine. The amount of adjuvant to administer will depend on the type and size of mammal. Optimal dosages may be readily determined by routine methods.

[0275] A further aspect of the present invention relates to a pharmaceutical composition, comprising at least one glycoprotein according to the invention. The preparation of medicaments comprising glycoproteins is well-known in the art. The preparation scheme for the final pharmaceutical composition and the mode and details of its administration will depend on the protein, the host cell, the nucleic acid and/or the vector employed.

[0276] It will be apparent to those of skill in the art that the therapeutically effective amount of polysaccharide or glycoprotein of this invention will depend, *inter alia*, upon the administration schedule, the unit dose of antibody administered, whether the polysaccharide or glycoprotein is administered in combination with other therapeutic agents, the immune status and health of the patient, and the therapeutic activity of the particular polysaccharide or glycoprotein.

[0277] The vaccine compositions and/or pharmaceutical preparations of the invention may be adapted for oral, parenteral or topical use and may be administered to the patient in the form of tablets, capsules, suppositories, solution, suspensions or any other suitable means or dosage form. In further aspects of the invention, the vaccine compositions and/or pharmaceutical preparations may be introduced into the subject to be immunized by any known method including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, or subcutaneous injection; or by oral, sublingual, nasal, anal, or vaginal, delivery. The pharmaceutically active compounds of the present invention, while effective themselves, can be formulated and administered in the form of their pharmaceutically acceptable salts, such as acid addition salts or base addition salts, for purposes of stability, convenience of crystallization, increased solubility, and the like. Vaccine compositions in an embodiment of the invention are administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Methods for intramuscular immunization are described by Wolff et al. (1990) Science 247: 1465-1468 and by Sedegah et al. (1994) Immunology 91: 9866-9870. Other modes of administration include oral and transdermal.

[0278] Vaccines of the invention can be administered as a primary prophylactic agent in, e.g., adults or in children, as a secondary prevention, after successful eradication of Gram-positive bacteria such as *S. aureus* in an infected host, or as a therapeutic agent in the aim to induce an immune response in a host to prevent infection by a Gram-positive bacterium such as *S. aureus*. The vaccines of the invention are administered in amounts readily determined by persons of ordinary skill in the art. The treatment may consist of a single dose or a plurality of doses over a period of time. For example, in some embodiments, it is expected that a typical dosage for humans of a vaccine of the present invention is about 1 to 25 μg of the oligosaccharide antigen, which will be bound to (and does not include the mass of) the protein carrier, in further embodiments about 1 μg to about 10μg of the polysaccharide antigen, and in still further embodiments about 2 μg of the polysaccharide antigen. In additional embodiments, the sugar/protein ratio in the glycoconjugate or the vaccine is about 1:5 to about 1:10. Optionally, a vaccine, such as a bioconjugate vaccine of the present invention, may include an adjuvant. Those skilled in the art will recognize that the optimal dose may be more or less depending upon the patient's body weight, disease, the route of administration, and other factors. Those skilled in the art will also recognize that appropriate dosage levels can be obtained based on results with known vaccines. The number of doses will depend upon the disease, the formulation, and efficacy data from clinical trials.

[0279] The vaccine compositions can be packaged in forms convenient for delivery. Delivery forms compatible with entry of the immunogenic component or fragment into the recipient mammal are preferred.

[0280] One embodiment of the invention is generally directed to recombinantly producing a vaccine for a Gram-positive organism in a Gram-negative organism by using a modified LPS biosynthetic pathway. This is accomplished by inserting into a host which comprises of nucleic acids encoding for an oligosaccharyltransferase and a protein and nucleic acids encoding for glycosyltransferases originating from at least two different organisms. This embodiment is directed to genetically engineering an organism based on a natural organism into which are inserted nucleic acids coding for (i) a protein; (ii) an oligosaccharyltransferase, and (iii) glycosyltransferases from at least two differing organisms.

[0281] In an example of such an embodiment, a glycosylated-protein product is produced for use as a vaccine for *Staphylococcus aureus*. The vaccine products of the invention are produced in a genetically modified *E. coli* host. *S. aureus* is a Gram-positive bacterium, and has a polysaccharide capsule. A vaccine product for this organism could be based on a glycosylated protein whose sugar section had a structure similar to this capsular polysaccharide.

[0282] In another aspect, the instant invention is directed to a novel bioengineering approach for producing immunogenic conjugate vaccines that provide advantages over classical chemical conjugation methods. In an embodiment, the approach involves *in vivo* production of glycoproteins in bacterial cells, for example, Gram-negative cells such as *E. coli*.

[0283] As known to a person of ordinary skill in the art, the production and purification of glycoconjugate can vary depending on the vaccine candidate and the combination of plasmids used. For example, which purification procedure to choose is known based upon the protein carrier, the sugar component of the glycoconjugate and the intended use of the purified vaccine candidate, for example, in animals or humans. For use in humans, for example, it is known that the HIS-tag, which would otherwise facilitate purification, would be removed.

[0284] It is to be understood that the term "or," as used herein, denotes alternatives that may, where appropriate, be combined; that is, the term "or" includes each listed alternative separately as well as their combination. As used herein, unless the context clearly dictates otherwise, references to the singular, such as the singular forms "a," an," and "the," include the plural, and references to the plural include the singular.

[0285] The invention is further defined by reference to the following examples that further describe the compositions and methods of the present invention, as well as its utility. It will be apparent to those skilled in the art that modifications, both to compositions and methods, may be practiced which are within the scope of the invention.

Examples

Example 1: Synthesis of CP5 and CP8 polysaccharide in *E. coli* cells

[0286] A goal of an embodiment of the invention is to produce the CP5 and CP8 antigenic polysaccharides in *E. coli*. As discussed above, we exploited in an novel way, surprising in view of the prior art, the fact that the CP and O-antigen production pathways functionally overlap, a fact which is represented in the structure of the RU (See FIGs. 1-4). The capsular glycans of CP5 and CP8 are polymers consisting of similar trisaccharide RUs of 2-Acetamido-2-deoxy-D-mannuronic acid (D-ManNAcA) and two 2-Acetamido-2,6-dideoxy galactose residues with D- and L-configurations (D- and L-FucNAc). The ManNAcA residues are linked differently in the two serotypes; additionally, the linkage between RUs in the polymerized glycan is different. In addition, there is an immunodominant O-acetyl modification at different positions in the two antigens (Jones, C. 2005. Revised structures for the capsular polysaccharides from *Staphylococcus aureus* types 5 and 8, components of novel glycoconjugate vaccines. Carbohydr Res 340:1097-106). The O11 antigen of *P. aeruginosa* LPS is similar in its structure to CP5 and CP8, as the O11 antigen of *P. aeruginosa* LPS contains [-3)–α-L-FucNAc–(1,3)–β-D-FucNAc–(1,2)–β-D-Glc-(1-] (FIG. 4). (Knirel, Y. A., V. V. Dashunin, A. S. Shashkov, N. K. Kochetkov, B. A. Dmitriev and I.L. Hofman. 1988. Somatic antigens of Shigella: structure of the O-specific polysaccharide chain of the Shigella dysenteriae type 7 lipopolysaccharide. Carbohydr Res 179: 51-60). The trisaccharide-RUs differ only in that the D-ManNAcA of *S. aureus* is replaced by a glucose unit, there is no O-acetyl modification in *P. aeruginosa* O11 LPS, and the difference in the linkage type between the 2nd and 3rd monosaccharide in the RU (FIG. 4).

[0287] To generate a genetic system able to synthesize the CP5 and CP8 glycans on UndPP, using the method of Dean et al., (Dean, C. R., C. V. Franklund, J. D. Retief, M. J. Coyne, Jr., K. Hatano, D. J. Evans, G. B. Pier, and J. B. Goldberg. 1999. Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. J Bacteriol 181:4275-4284), we modified the *P. aeruginosa* O11 O-antigen gene cluster from strain PA103. The genes encoding the biosynthetic machinery for synthesis of the stem structure consisting of UndPP-D-FucNAc-L-FuncNAc were complemented with the *S. aureus* enzymes required for the completion of the *S. aureus* glycan (FIG. 1-4), which was also a novel use of this process. Therefore, using the method of Dean et al., all the genetic elements from *P. aeruginosa* PA103 required for the UndPP-FucNAc-FucNAc biosynthesis were expressed. The gene encoding the glycosyltransferase adding the third sugar was deleted and replaced by the corresponding genes from the cap5 or 8 clusters form *S. aureus* Mu50 (CP5) and MW2 (CP8) with slight modifications.

[0288] The genes encoding the enzymes synthesizing the specific residues for the *S. aureus* capsular polysaccharide were integrated step by step into the O11 background according to the functions of the genes predicted by Sau et al. (Sau, S., N. Bhasin, E. R. Wann, J. C. Lee, T. J. Foster, and C. Y. Lee. 1997. The *S. aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. Microbiology 143: 2395-405.; O'Riordan, K. and J. C. Lee. 2004. *Staphylococcus aureus* capsular polysaccharides. Clin Microbiol Rev 17(1): 218-34). Such steps are explained below.

[0289] The *cap5I/cap8H* gene product was predicted to be the glycosyltransferase that adds the ManNAcA to UndPP-D-FucNAc-L-FuncNAc of the RU forming a linkage specific for each serotype (Sau, S., N. Bhasin, E. R. Wann, J. C. Lee, T. J. Foster, and C. Y. Lee. 1997. The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. Microbiology 143: 2395-405.). To prove this, the activity of Cap5I and Cap8H was analyzed in *E. coli* in presence of a plasmid conferring production of the

P. aeruginosa O11 O-antigen. Cells expressing the O11 cluster synthesize the O11 O-antigen first on UndPP, from where it is transferred to lipid A core by the *E. coli* enzyme WaaL, the O-antigen ligase, forming O11 specific lipopolysaccharide (LPS) (Goldberg, J. B., K. Hatano, G. S. Meluleni and G. B. Pier. 1992. Cloning and surface expression of *Pseudomonas aeruginosa* O antigen in *Escherichia coli*. Proc Natl Acad Sci U S A 89(22): 10716-20). To synthesize this lipopolysaccharide, the O11 O-antigen cluster from *P. aeruginosa* PA103 was cloned into pLAFR1 (SEQ ID NO: 1). Then the *wbjA* gene encoding the glucosyltransferase, the enzyme adding the third sugar to the O11 RU, was deleted by transposon mutagenesis. The mutated cluster (O11 *wbjA*::Tn50<*dhfr-1*>) was further modified by homologous recombination to eliminate the polymerase activity of the *wzy* gene, forming O11 *wbjA*:: Tn50<*dhfr-1*> *wzy*::*cat*, which denotes the mutated SEQ ID NO: 1, in which the genes for the glycosyltransferase wbjA and the wzy polymerase of the O11 gene cluster were inactivated. This modified cluster was expressed in W3110 Δ*wecA* cells, extracts were treated with proteinase K and analyzed by SDS PAGE and silver staining, according to the method disclosed in Tasi, et al. (Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119:115-9). The results are provided in FIG. 5A, showing silver staining of W3110 Δ*wecA* extracts expressing the mutated O11 cluster from pLAFR1 as described herein. The second line indicates the genes expressed from the inducible plasmid pEXT22. Asterisks indicate synthesized and codon optimized genes. Different relevant glycoforms are indicated with arrows.)

[0290] Analysis resulted in two major bands in the gels (FIG. 5A, lane 1). The signals correspond to the unmodified lipid A core (FIG 5A, lower band) and LPS consisting of lipid A core and two FucNAc residues as expected in a truncated O11 RU. Upon expression of a *wbjA* wildtype copy from a separate, IPTG inducible plasmid, the upper band shifted to a slower electrophoretic mobility, indicating the addition of a glucose residue to the truncated O11 LPS (FIG 5A, lane 2). When the predicted *S. aureus* glycosyltransferases Cap5I (lane 4) and Cap8H (FIG 5A, lane 3) were expressed in trans instead of WbjA, a similar shift of the glycosylated lipid A core signal was observed, indicative of addition of a monosaccharide possibly even larger than glucose, most probably being ManNAcA. This data proves that *S. aureus* glycosyltransferases can elongate

UndPP-D-FucNAc-L-FuncNAc glycolipid that has been synthesized by activity of *P. aeruginosa* enzymes.

[0291] In this way it was also confirmed that a prerequisite for *S. aureus* RU assembly in *E. coli* is the provision of UDP-ManNAcA, because the biosynthetic machinery is present in the *S. aureus* CP5/8 clusters but not in the O11 O-antigen cluster of *P. aeruginosa*. All other required nucleotide activated sugars are either provided by housekeeping functions of *E. coli* and the O11 O-antigen cluster of *P. aeruginosa*. *E. coli* is known to produce UDP-ManNAcA, the substrate for the ManNAcA glycosyltransferase, by expression of *wecB* and *wecC*. Those genes are constitutively expressed in the cluster responsible for enterobacterial common antigen (ECA) biosynthesis (Meier-Dieter, U., R. Starman, K. Barr, H. Mayer, and P. D. Rick. 1990. Biosynthesis of enterobacterial common antigen in *Escherichia coli*. J Biol Chem 265:13490-13497). The functional homolog for UDP-ManMAcA biosynthesis found in the CP cluster of *S. aureus* were found to complement the activities of *wecBC* as reported earlier (Kiser, K. B., N. Bhasin, L. Deng and J. C. Lee. 1999. *Staphylococcus aureus* cap5P encodes a UDP-N-acetylglucosamine 2-epimerase with functional redundancy. J. Bacteriol 181(16): 4818-24). This shows that the production of the CP antigens in *E. coli* relies on the functional expression of the *wecBC* genes in the host strain. Thus, to provide UDP-ManNAcA as substrate for Cap5I and Cap8H in a recombinant system, it was confirmed that WecB and WecC have to be expressed. In such a system, any prokaryotic strain expressing the enterobacterial common antigen like *E. coli* wildtype strain can be used, e.g. W3110 based cell types with or without a *wecA* deletion and with or without additional *wzzE* deletion.

[0292] Further elongation of the *S. aureus* capsular polysaccharide is thought to be required for maximal immunological activity of the glycan. The *cap5J/cap8I* genes encode the *wzy* homologs polymerizing the repeating units, and *cap5K/cap8K* encodes the flippase translocating the UndPP-bound trisaccharide from the cytoplasmic to the periplasmic side of the membrane. *Cap5H/cap8J* encodes the O-acetyltransferase modifying the L-FucNAc at position 3' or the ManNAcA at position 4' of the RU (Bhasin, N., A. Albus, *et al.* (1998). "Identification of a gene essential for O-acetylation of the *Staphylococcus aureus* type 5 capsular polysaccharide." Mol Microbiol 27(1): 9-21. The acetylation is an important determinant discriminating the immunological reactivity of the polysaccharide (Fattom, A. I., J. Sarwar, L. Basham, S. Ennifar, and R. Naso. 1998. Antigenic determinants of *S. aureus type* 5 and type 8 capsular polysaccharide vaccines. Infect Immun 66:4588-92). To show that the RUs could be elongated and acetylated, the *S. aureus* enzymes responsible for polymerization and O-acetylation were expressed from separate plasmids in presence of the mutated O11 cluster. Extracts from W3110 Δ*wecA* cells expressing the O11 *wbjA*::Tn50<*dhfr-1*> *wzy*::*cat* cluster and different genes of the CP5 cluster were treated with proteinase K and analyzed by SDS PAGE, electrotransfer followed by immunoblotting using an anti CP5 sugar (obtained from J. C. Lee at the Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). FIG. 5B shows the results of immunodetection of proteinase K treated *E. coli* extracts separated by SDS PAGE and electrotransfer using the anti CP5 antiserum. All extracts analyzed contained a *P. aeruginosa* O11 cluster with deletions of the *wbjA* and partially (indicated by an asterisk) the *wzy* genes expressed from the pLAFR plasmid as described herein, and two more plasmids (pEXT22, pACT3) expressing different Cap5 proteins (as indicated) that enable CP5 polymerization and O acetylation in these cells. Experimental details such as inducer concentrations and expression culture incubation temperatures are indicated.

[0293] In FIG. 5B, the results show ladder like signals typical for an O-antigen polymer in a higher molecular weight range. The different bands represent different numbers of linearly polymerized RUs on LPS or on UndPP, both of which are stable towards proteinase K digestion. Different intensities of the ladder like structure in presence or absence of the O-acetyltransferase were observed. Whereas strong signals were detected in the presence of *cap5H* (FIG 5B, lanes 1-4), they were virtually absent in lanes without *cap5H* (FIG 5B, lanes 5, 6). This means that O-acetylation either increases recognition by the specific antiserum, or that it enhances polymerization activity by either accelerating flipping or making polymerization as such more efficient or by inducing more RU production. The *cap5H* gene is functional when expressed from different backbone plasmids (FIG 5B, lanes 1, 2 and 3, 4), although signal intensity is stronger when *cap5H* is expressed alone from a separate plasmid (compare FIG. 5B lane 1 to lane 3 and FIG. 5B, lane 2 to lane 4). It is surprising and remarkable that the less IPTG was used for induction of the *S.*

aureus genes, the stronger the signals (compare FIG. 5B, lane 1 to land 2 and FIG. 5B, lane 3 to lane 4).

Example 2: Synthesis of CP5 and CP8 polymer on lipid in *E. coli* cells

[0294] As high expression of the *cap5* specific genes lead to lower polymer formation, an alternative expression system for the recombinant glycans was constructed to address this problem. In detail, in a novel approach unexpected in light of the prior art, the *P. aeruginosa* glucosyltransferase (*wbjA*) and the polymerase (*wzy*) of O11 were replaced by the genes encoding the CP5/8-specific elements from the capsular gene cluster of *S. aureus* Mu50/MW2 (*cap5/8HIJK* and parts therof) producing a single, chimeric gene cluster composed of *P. aeruginosa* O11 and *S. aureus* CP5 or CP8 genes (FIG. 6). The construct contained the specific genes of *S. aureus*. Each was tagged for expression detection and each contained an introduced ribosomal binding site, and was followed by a chloramphenicol resistance cassette (*cat*) for selection of recombined clones resulting in SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4, according to the method of Datsenko, et al. (Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5).

[0295] FIG. 6 depicts an embodiment of a strategy of the present invention for construction of chimeric O11/CP5 and O11/CP8 gene clusters of the present invention. The *S. aureus* CP5 and CP8 CP clusters (top) and the *P. aeruginosa* PA103 *rfb* cluster (O11, middle) are represented as published (Dean, C. R., C. V. Franklund, J. D. Retief, M. J. Coyne, Jr., K. Hatano, D. J. Evans, G. B. Pier, and J. B. Goldberg. 1999. Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. J Bacteriol 181:4275-84; Sau, S., N. Bhasin, E. R. Wann, J. C. Lee, T. J. Foster and C. Y Lee. 1997. The *S. aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. Microbiology 143 (Pt 7): 2395-405). The homologous functions of the genes are described below. Complete forward diagonals indicate the genes responsible for synthesis of the D-FucNAc-L-FucNAc disaccharide on UndPP in the two organisms; dots indicate the glycosyltransferase genes adding the third monosaccharide to the RU. *Wzx*-like flippase genes are indicated by broken forward diagonals, the *wzy*-like RU polymerase genes are indicated by broken back diagonals. The CP5 cluster does not contain a Wzz length regulator (empty arrow), but a set of three genes composing the export machinery for capsular polysaccharide which includes the length regulator function in *S. aureus* (empty arrows). The O acetyl transferase gene, indicated by complete forward diagonals, is unique to the CP cluster. The genes required for UDP-ManNAcA biosynthesis in *S. aureus* are indicated in black. They are not required for production of the *P. aeruginosa* O-antigen. The genes responsible for the structural differences of the O11, CP5 and CP8 polysaccharides are clustered together in the beginning (O11: *wbjA* and *wzy*) or middle (CP5/8: *cap5/8HIJK*) of the respective gene clusters. The CP8 cluster is almost identical to the CP5 cluster considering length and DNA sequence, except for the middle part (cap5/8HIJK) conferring structural specificity. The chimeric cluster was constructed by replacing *wbjA* and *wzy* genes of a plasmid borne O11 cluster with the specificity part of the CP5 (or CP8) cluster (*cap5/8HIJK*) and a chloramphenicol acetyltransferase cassette represented by the empty arrow labeled cat (*cat*, for selection) by homologous recombination and classical clonings, resulting in SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4. Asterisks at the broken arrows indicate incomplete gene sequences used for homologous recombination. The resulting two chimeric clusters are shown in the bottom panel, representing the DNA of SEQ ID NO: 3 and SEQ ID NO: 4.

[0296] To prove that the chimeric CP5 and CP8 of the present invention surprisingly assembles the correct RU on UndPP and assures that the repeating units are polymerized, proteinase K digestion of *E. coli* cells (W3310 Δ*wecA*) containing the full length chimeric clusters were separated by SDS-PAGE. Specifically, cells with a plasmid either containing or lacking the chimeric CP5 gene cluster (FIG. 7A) or the chimeric CP8 gene cluster (FIG. 7B) on the pLAFR plasmid were treated with Proteinase K, separated by SDS-PAGE and lipids were visualized by either silver staining (left panel in FIGs. 7A and 7B) or immunodetection with anti CP5 or CP8 antiserum after electrotransfer to nitrocellulose membranes (right panel in FIG. 7A and B)). Constructs lacking (SEQ ID NO: 2) and containing (SEQ ID NO: 3) the flippase gene *cap5K* were tested. The former was found to be less active in CP5 LPS production.

[0297] After electrotransfer and immunodetection with anti CP5 specific serum, extracts expressing the entire chimeric CP5 clusters show a ladder like signal similar to endogenous O-antigen structures from *E. coli* probed with their autologous serum (FIG. 7A, last two lanes on the right). This strongly suggests that the CP5 repeating units are polymerized, that there is a preferred polymer length, and that the CP5 antigen is transferred to lipid A core in these cells. The same extracts were visualized by silver staining after SDS PAGE (FIG. 7A, on the left side of the figure, the two lanes on the right labeled as: chimeric CP5 (w/o cap5K) and chimeric CP5 showing that indeed LPS is formed consisting of the lipid A core of *E. coli* decorated with the CP5 O-antigen-like structure. Intensity differences were obtained from extracts originating from cells that expressed the CP5 chimeric cluster with or without the *cap5K* flippase gene. Comparison of the two extracts shows that Cap5K expression considerably increases the polymer production (compare middle and right lanes in both panel of FIG. 7A).

[0298] As shown in FIG. 7B, the same results were observed with a CP8 chimeric cluster. Cells containing a plasmid either containing or lacking the chimeric CP8 gene cluster on the pLAFR plasmid were treated with Proteinase K, separated by SDS PAGE and lipids were either detected by silver staining (left panels) or immunodetection with anti CP8 antiserum after electrotransfer to nitrocellulose membranes (right panel). CP8 chimeric construct containing the flippase gene *cap8K* corresponds to SEQ ID NO: 4.

[0299] A further novel and surprising extension of the invention was developed by changing the plasmid backbones used for maintenance and expression of the chimeric cluster in *E. coli*. The resistance cassette in pLAFR1 containing the chimeric CP5 cluster was changed from Tet to Kan. Additionally the entire CP5 chimeric cluster containing the *cap5K* was subcloned into plasmid pDOC-C, according to the method of Lee et al. (Lee, D. J., L. E. Bingle, K. Heurlier, M. J. Pallen, C. W. Penn, S. J. Busby and J. L. Hobman. 2009. Gene doctoring: a method for recombineering in laboratory and pathogenic *Escherichia coli* strains. BMC Microbiol 9: 252) and pACYC177 (GeneBank accession #X06402).

[0300] As shown in FIGs. 8A and 8B, all of these plasmids conferred CP5 polymer production as analyzed by SDS PAGE, electrotransfer and immunodetection with anti CP5 specific antiserum. In FIG 8A, total cell extracts from cells containing different chimeric clusters were treated with Proteinase K and analyzed by SDS PAGE and silver staining. The plasmids contain different *S. aureus* specific genes and different resistance genes used for antibiotic selection are indicated: Tetracycline (Tet) and HIJ, SEQ ID NO: 2; Tet HIJK, SEQ ID NO: 3, Tet and no genes, empty plasmid control, numbers correspond to molecular weight markers. Lanes labeled Kanamycin (Kan) contains a variation of SEQ ID NO: 3 in which the tetracycline resistance cassette is replaced by a kanamycin resistance gene.

[0301] In FIG. 8B, the host strain was *E. coli* W3110 Δ*wecA*, as in FIG. 8A. The left lane in FIG. 8B corresponds to the molecular weight marker as in FIG. 8A. In FIG. 8B, total cell extracts from cells containing different chimeric clusters were treated with Proteinase K and analyzed by SDS PAGE and silver staining (left panel) and by anti CP5 immunoblotting after electrotransfer (right panel). The plasmids used contain the chimeric CP5 cluster indicated in SEQ ID NO: 3 either present in a modified pLAFR1 plasmid backbone containing a Kanamycin cassette instead of tetracycline (see FIG. 8A) or in pACYC containing a chloramphenicol resistance cassette.

[0302] In addition different promoters were tested to express the chimeric O11-CP5 LPS. In these tests, the host strain was *E. coli* W3110 Δ*wecA* carrying the chimeric CP5 cluster. In this strain, the chimeric cluster replaced *wecAwzzE* genes. Total cell extracts from cells containing different chimeric clusters expressed from pLAFR1 were treated with Proteinase K and analyzed by SDS PAGE and anti CP5 immunoblotting after electrotransfer. The plasmids contained O11 clusters where *wbjA* and *wzy* were replaced by different *S. aureus* specificity genes (with a *cat* cassette) as indicated below the lanes in FIG. 9. In addition, the DNA in front of the *cap5* specificity genes was changed and the effect on lipid glycosylation was analyzed. The effect of these different promoter regions was analyzed as depicted in FIG. 9. *Wzz/wzx* denotes the original genes (see FIG. 6) in front of the *cap* genes after the initial homologous recombination (FIG. 9 corresponding to the first two lanes). These two genes were removed (FIG. 9 corresponding to the three lanes in the middle) and replaced with the 0.6 kb region (PO121) (FIG. 9 corresponding to the three last lanes) in front of the *E. coli* O121 O-antigen cluster encoding a strong promoter sequence. Lanes denoted *wzz/wzx* and HIJ in FIG. 9 were derived from cells expressing SEQ ID NO: 2, lanes denoted *wzz/wzx* and HIJK derive from SEQ ID NO: 3. In FIG. 9, the molecular weight markers are indicated on the left of the gel frame.

[0303] As indicated FIG. 9, the results showed that a relevant promoter activity resides in the *wzx* gene (FIG. 9 first two lanes- wzz/wzx) and that it can be functionally replaced by a constitutive promoter from *E. coli*, e.g. the serovar O121 wb promoter (PO121 last three lanes in FIG. 9), without losing LPS production. Taken together, these results mean that the O11 and *S. aureus* elements for O11 O-antigen and CP5 capsular polymer production as described herein can be combined in many different *E. coli* expression systems resulting in production of recombinant *S. aureus* polysaccharide.

[0304] These results showed for the first time the production in *E. coli* of a capsular polysaccharide structure originating from a Gram-positive organism. This means that it was possible, contrary to prior art and conventional expectations, to combine the enzymes of the O11 cluster and the enzymes of *S. aureus* cap cluster to build up a chimeric polysaccharide, i.e. that the enzyme work together on the same structure *in vivo*.

Example 3: Molecular structure confirmation of the recombinant glycans

[0305] To confirm the activity of the chimeric CP5/O11 cluster in *E. coli* on a molecular level, a novel method allowing the analysis of UndPP linked sugars by using fluorescent labeling of the sugar at reducing end with 2-Aminobenzamide (2-AB) was developed. To enhance the analysis resolution, chimeric clusters were used containing deletions that increased the amount of unpolymerized RUs. Glycolipids from different *E. coli* cells expressing the chimeric cluster contained in the pLAFR1 plasmid and lacking the cap5K flippase (SEQ ID NO: 2) were analyzed as described below.

[0306] To extract UndPP-linked glycans, *E. coli* cells were washed with 0.9% NaCl and lyophilized. The dried cells were extracted once with 30 ml organic solvent (85 to 95 % Methanol = M). The lyophilized cell pellet was further extracted twice with 5 ml Chloroform:Methanol:Water (C:M:W = 10:10:3; v/v/v). The (M) extract was converted with chloroform and water to a final ratio of 3:48:47 (C:M:W). The 10:10:3 (C:M:W) extract was converted to a two-phase Bligh/Dyer (Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37(8): 911-7) system by addition of water, resulting in a final ratio of 10:10:9 (C:M:W). Phases were separated by centrifugation and the upper aqueous phase was kept for further processing.

[0307] To purify the extracted glycolipids, aqueous phase was subjected to a tC_{18} Sep-PAK cartridge. The cartridge was conditioned with 10 ml methanol, followed by equilibration with 10 ml 3:48:47 (C:M:W). After loading of the sample, the cartridge was washed with 10 ml 3:48:47 (C:M:W) and eluted with 5 ml methanol and 5 ml 10:10:3 (C:M:W). The combined elutions were dried under N_2. The glycolipid samples were hydrolyzed by dissolving the dried samples in 2 ml n-propanol:2 M trifluoroacetic acid (1:1), heating to 50 °C for 15 min, and then evaporating to dryness under N_2 (Glover, K. J., E. Weerapana and B. Imperiali. 2005. In vitro assembly of the UndPP-linked heptasaccharide for prokaryotic *N*-linked glycosylation. Proc Natl Acad Sci U S A 102(40): 14255-9). The dried samples were labeled with 2-AB and the glycan cleanup was performed using the paper disk method as described (Bigge, J. C., T. P. Patel, J. A. Bruce, P. N. Goulding, S. M. Charles, R. B. Parekh. 1995. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal Biochem 230(2): 229-38; Merry, A. H., D. C. Neville, L. Royle, B. Matthews, D. J. Harvey, R. A. Dwek and P. M. Rudd. 2002. Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis. Anal Biochem 304(1): 91-9). The 2-AB labeled glycans were separated by HPLC using a GlycoSep-N normal phase column according to Royle *et al.* but modified to a three solvent system (Royle, L., T. S. Mattu, E. Hart, J. I. Langridge, A. H. Merry, N. Murphy, D. J. Harvey, R. A. Dwek, P. M. Rudd. 2002. An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. Anal Biochem 304(1): 70-90). Solvent A was 10 mM ammonium formate pH 4.4 in 80 % acetonitrile. Solvent B was 30 mM ammonium formate pH 4.4 in 40 % acetonitrile. Solvent C was 0.5 % formic acid. The column temperature was 30 °C and 2-AB labeled glycans were detected by fluorescence (excitation λex = 330 nm, emission λem = 420 nm). Gradient conditions were a linear gradient of 100 % A to 100 % B over 160 min at a flow rate of 0.4 ml/min, followed by 2 min 100 % B to 100 % C, increasing the flow rate to 1 ml/min. The column was washed for 5 min with 100 % C, returning to 100 % A over 2 min and running for 15 min at 100 % A at a flow rate of 1 ml/min, then returning the flow rate to 0.4 ml/min for 5 min. Samples were injected in water.

[0308] Dried fractions were resuspended in 5ul 10% acetonitrile (ACN), 0.1% trifluoro acetic acid (TFA) and mixed 1:1 with matrix solution (40mg/ml DHB in 50% ACN, 0.1% TFA) on the target plate. MS and MS/MS data were manually acquired in the positive ion mode on an Ultraflex-II MALDI-ToF/ToF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). MS/MS were obtained using the LIFT method. A standard peptide mixture (Bruker Daltonik GmbH) was used for external calibration. Spectra were exported using the Flex Analysis software (Bruker Daltonik GmbH) and manually analyzed.

[0309] Methanol extracts from *E. coli* W3110 Δ*wecA* (CP5) containing plasmids with (thick line) or without (thin, dashed line) the chimeric clusters were purified over tC18 cartridges and analyzed by normal phase HPLC. The fractions corresponding to the peaks shown in FIG. 10A found at 37', 40' and 45' elution were analyzed by MALDI-MS/MS. Samples eluting at 37 and 40 minutes were identified as recombinant CP5 RUs with and without the O-acetyl group attached, respectively. Sample eluting at 45 minutes was identified as non-acetylated *S. aureus* RU structure elongated by one deoxy-N-acetylhexosamine (as shown in FIG. 11E). In the CP5 chimeric cluster, *cap5HIJ* replaced the *wbjA* and *wzy* genes of the O11 cluster on pLAFR. The replacement contained the *cat* cassette in addition to the *cap5HIJ* genes (SEQ ID NO: 2).

[0310] Methanol extracts from *E. coli* W3110 Δ*wecAwzzE* containing plasmids with (thick line) or without (thin, dashed line) the chimeric cluster were purified over tC18 cartridges and analyzed by normal phase HPLC. FIG. 10B shows the results of HPLC analysis of recombinant RU of CP8 produced using a chimeric cluster (SEQ ID NO: 4 without polymerase). Peaks specific for cells expressing the recombinant sugar were identified at 23', 32', 38' and 45 of elution, collected and analyzed by MALDI-MS and MALDI-MS/MS. In the CP8 chimeric cluster, *cap8HJK* replaced the *wbjA* and *wzy* genes of the O11 cluster, i.e. a construct without the polymerase to accumulate single RU for analysis. The replacement contained the *cat* cassette in addition to the *cap* genes.

[0311] FIG. 11A shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP5 cluster of the present invention in *E. coli* eluting at 37 minutes. The major mass m/z=772 ($[M+H]^+$) was selected and analyzed by MS/MS, which shows a fragmentation pattern consistent with the acetylated CP5 RU structure that was expected in light of the invention disclosed in this specification. The O-acetylated species are characterized by a specific loss of 42 plus the mass of the monosaccharide FucNAc (dHexNAc(OAc)) at the middle position of the RU. Fragment ions are indicated according to the nomenclature of the consortium for functional glycomics, CFG (www.functionalglycomics.org/static/consortium/Nomenclature.shtml). 2-AB, 2-aminobenzamide. The legend for the fragment ions is given in the inset of FIG. 11A.

[0312] FIG. 11B shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP5 cluster of the present invention in *E. coli* eluting at 40 minutes. The major mass of m/z= 730 ($[M+H]^+$) was selected and analyzed by MS/MS, which shows fragmentation ion series consistent with the non-acetylated CP5 RU structure that was expected in light of the invention disclosed in this specification. 2-AB, 2-aminobenzamide. The legend for the fragment ions is given in the inset of FIG. 11B.

[0313] FIG. 11C shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP8 cluster of the present invention in *E. coli* eluting at 32 minutes. A major mass of m/z=794 ($[M+Na]^+$) was selected and analyzed by MS/MS, which shows fragmentation ion series consistent with the acetylated CP8 RU structure that was expected in light of the invention disclosed by this specification. The O-acetylated species are characterized by a specific loss of 42 plus the mass of the monosaccharide ManNAcA (HexNAcA(OAc))at the outermost position of the RU. Fragment ions are indicated according to the nomenclature of the CFG. 2-AB, 2-aminobenzamide. The legend for the fragment ions is given in the inset of FIG. 11C.

[0314] FIG. 11D shows the results of MALDI-MS/MS analysis of the specific peak generated by expression of an embodiment of the chimeric CP8 cluster of the present invention in *E. coli* eluting at 38 minutes. The mass of m/z= 730 ($[M+H]^+$) was selected and analyzed by MS/MS, which shows fragmentation ion series consistent with the non-acetylated CP8 RU structure that was also expected in light of the invention disclosed in this specification. Additional analysis showed that the later eluting peaks (shown in FIG. 10A at 40 min and FIG. 10B at 38 min) contain the non-O-acetylated trisaccharides of CP5 and 8 RUs. Fragment ions are indicated according to the nomenclature of the CFG. 2-AB, 2-aminobenzamide. The legend for the fragment ions is given in the inset of FIG. 11D.

[0315] MS results showed that the masses and fragmentation ion series are in agreement with the molecular structure of the CP5 RU oligosaccharide with the O acetylation of the middle FucNAc residue (i.e., the peak at 37' in FIG. 10A and in FIG. 11A) or without the O acetylation of the middle FucNAc residue (i.e., the peak 40' in FIG. 10A and in 11B). The signal at 45 minutes in FIG. 10A was identified as a tetrasaccharide, which is further analyzed below. The same analysis was repeated with the chimeric CP8 cluster that lacked the polymerase gene. In such extracts, signals consistent with the O-acetylated RU structure expected in light of the invention disclosed in this specification were found at 23' and 32' of elution, as shown FIGs. 10B and 11C. The presence of two different elution times for the same glycan sequence as identified by MALDI-MS/MS indicates an O-acetyl migration event taking place during sample preparation. Non-acetylated RUs were identified for CP5 and CP8 extracts at 40' and 38', as shown in FIGs. 11B and D, respectively. The CP5 and CP8 RU structures were present in different *E. coli* strains, including for example, W3110, W3310 ΔwecA, W3110 Δ*wecAwzzE*, and W3110 Δ*wecAwzzE* Δ*waaL*.

Example 4: Improvement of the repeating unit structure and its analysis.

[0316] The HPLC peak shown in FIG. 10B eluting at 45 minutes, derived from *E. coli* cells expressing the chimeric CP8 cluster (SEQ ID NO: 4) but lacking the wzy polymerase gene cap8I, was also analyzed by MALDI-MS/MS. The most intense ion in the full scan MS was m/z=939 ($[M+H]^+$) and sequence analysis was performed by MS/MS. The results of this MS/MS analysis are shown in FIG. 11E, and present a fragmentation ion series consistent with the non acetylated *S. aureus* capsular RU extended by a mass of a deoxy-N-acetylhexosamine at the non-reducing end, as expected in light of the invention disclosed in this specification. Fragment ions corresponding to the hypothetical structures are indicated according to the nomenclature of the CFG above the peaks. 2-AB, 2-aminobenzamide. The legend for the fragment ions is given in the inset of FIG. 11E.

[0317] The result shown in FIG. 11E suggested that an *E. coli* glycosyltransferase was able to modify the ManNAcA residue of the CP8 RU. Such an altered RU most probably would not be polymerized by cap8I. Analysis of the glycosyltransferase specificities in the *E. coli* host W3110 indicated that an enzyme from the ECA cluster may interfere with the recombinant sugar, specifically the wecF gene product, a putative 4-N-acetylfucosamine transferase. WecF naturally adds a 4-N-acetylfucosamine onto ManNAcA comprised in ECA, most likely the enzyme could also elongate CP8 and CP5 RU.

[0318] To solve this problem, another novel approach was developed. Specifically, genes of the ECA cluster located downstream of the *wecC* gene including *wecF* were deleted. This was accomplished using the method described by Datsenko et al. (Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products." Proc Natl Acad Sci U S A 97(12): 6640-6645). Different *E. coli* expression hosts were deleted in the *waaL* and *rmlB-wecG* gene regions and in some strains in *wecA-wzzECA* as well. Sep-PAK Purified extracts (Methanol and 10:10:3 extracts) from these mutated cells expressing the polymerase mutant CP8 chimeric cluster were analyzed by normal phase HPLC as described above.

[0319] FIG. 11F presents the results of HPLC analyses of methanol extracts from *E. coli* W3110 *ΔwaaL* cells expressing the polymerase mutant of SEQ ID NO: 4 (thin, dashed line) compared to cells with an additional deletion of the ECA cluster genes rmlB-wecG (W3110 *ΔwaaL ΔrmlB-wecG::cat*) (thick line). Extracts were purified over tC18 cartridges and analyzed by normal phase HPLC. As shown in FIG. 11F, the major peak appearing at 45' in FIG. 10B was absent resulting in specific peaks for the acetylated and non acetylated CP8 RUs (FIG. 11F) indicating that one of the ECA glycosyltransferases – most probably *wecF* – is responsible for the aberrant elongation phenotype. Similar results were obtained when the CP5 chimeric cluster was tested in different strains. This implies that deleting *E. coli* borne glycosyltransferases and enzymes required for nucleotide activated sugar biosynthesis is a possible strategy for optimizing quality and quantity of recombinantly produced polysaccharides in *E. coli*. Target enzymes most likely would be encoded in the O-antigen cluster, the ECA cluster, and the colanic acid or capsule clusters.

[0320] Further evidence for the quality of the recombinant polysaccharide linked to UndPP was obtained from an optimized normal phase HPLC analysis of Sep-PAK purified, fluorescently labeled glycolipid extracts from chromosomally optimized expression hosts as described above. For optimal performance of the Sep-PAK columns for purification of charged CP5 and CP8 oligo- and polysaccharide-linked lipids, tert-butyl ammonium phosphate (TBAP) was added to the extracts before loading on the Sep-PAK cartridges. As reported by Trent, et al., the cation of this salt improves column binding of charged compounds by shielding negative charges with hydrophobic butyl chains (Trent, M. S., A. A. Ribeiro, *et al.* (2001). "Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant Salmonella typhimurium and *Escherichia coli*: structural characterization and transfer to lipid A in the periplasm." J Biol Chem 276(46): 43132-43144.). This optimized method was applied to the CP5 and CP8 samples obtained by methanol extraction from cells expressing CP5 or CP8 chimeric clusters containing a polymerase.

[0321] FIG.11G provides the results of HPLC analysis showing the full CP5 glycan repertoire present on UndPP in *E. coli* cells. Methanol extracts from *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* either expressing the chimeric CP5 cluster SEQ 3 (solid line) or an empty plasmid control (dashed line) were solid-phase extracted on Sep-PAK cartridges and treated with mild acid to hydrolyse sugars from UndPP. The resulting material was reacted with 2AB by reductive amination to label reducing ends of the glycans and analyzed by normal phase HPLC. Signals present in the solid line but not in the dashed line represent CP5 specific material. Capital letters indicate peaks containing polymers of the acetylated and/or non-acetylated CP5 RU as identified by MALDI-MS/MS of the collected fractions. The legend of FIG. 11G indicates the proposed molecular structures as deduced from MS/MS analysis. It should be noted that acetylated and non-acetylated RU polymers shown for MS/MS confirmed structures of the same polymerization degree group together in the chromatogram as indicated by thick bars. Capital letters show the following lengths: A and B: one RU; C, D and E: two RUs; F and G: three RUs; and H: four RUs. The broad peak between 95' and 125' in FIG. 11G most probably represents 5 or more polymerized RUs not resolved by the column.

[0322] FIG. 11H presents further HPLC results, showing acetylated CP5 glycans and RU homogeneity. To prepare this HPLC analysis, 2AB labeled glycan samples of *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* expressing the chimeric CP5 cluster SEQ ID NO.: 3 (prepared according to the procedures described above with reference to FIG. 11G) were treated with NaOH in aqueous solution and re-labeled. As showing in FIG. 11H, samples before (dashed) and after (solid line) alkali treatment were analyzed by HPLC. Numbers in FIG. 11H indicate the putative numbers of RUs in the corresponding peaks. It should be observed that, in FIG. 11H, the acetylated peaks shown in FIG. 11G unify in the signal from non-acetylated polymer, and that deacetylation resolved the RU units in the elution times after 95 minutes.

[0323] FIG. 11I provides the results of HPLC analysis showing the CP8 glycan repertoire present on UndPP in *E. coli* cells. Methanol extracts from *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* either expressing the chimeric CP8 cluster SEQ ID NO.: 4 (solid line) or an empty plasmid control (dashed line) were solid-phase extracted on Sep-PAK cartridges and treated with mild acid to hydrolyse sugars from UndPP. The resulting material was reacted with 2AB by reductive amination to label reducing ends of the glycans and analyzed by normal phase HPLC. Signals present in the solid line but not in the dashed line represent CP8 specific material. Putative structures of acetylated and/or non-acetylated CP8 RU as identified by MALDI-MS/MS of the collected fractions are indicated. Note that as in the HPLC results with CP5 shown in FIG. 11G, acetylated and non acetylated CP8 RU polymers of the same polymerization degree group together in the chromatogram of FIG. 11H as indicated by thick bars. Material detected after 110' represents longer CP8 polymers.

[0324] FIG. 11J presents further HPLC results, showing deacetylation of CP8 glycans and RU homogeneity. 2AB labeled glycan samples from *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* expressing the chimeric CP8 cluster SEQ ID NO.: 4 were treated with NaOH in aqueous solution and re-labeled. Samples before (dashed) and after (solid line) alkali treatment were analyzed by HPLC. Numbers indicate the putative numbers of RUs in the corresponding peaks. It should be noted that the acetylated peaks largely vanish and that signals of non-acetylated polymer increase, and that deacetylation resolved the RU units in the elution times after 110 minutes.

[0325] FIGs. 11H and 11J show HPLC results indicative of the characteristic ladder-like banding pattern of O-antigens when alkali treatment was performed on these CP5 and CP8 samples to remove the acetylation modifications from the oligo- and polysaccharides., The results show discrete sharp peaks with constantly decreasing elution time increments. This implies that such analyzed carbohydrate chains are linear polymers composed of identical RUs. This data shows that the recombinant CP5 and CP8 sugars produced in *E. coli* are regularly polymerized and partially acetylated. Non-acetylated CP5 and CP8 polymers elute similarly from the HPLC column as expected from their similarity in structure; however the normal phase chromatography also reveals differences: for example, CP5 polymerizes to a lesser extent than CP8, and acetylation is more frequent in CP5; in the RU lengths above 4, CP5 has a clear preference for making polymers of 7 RUs, whereas CP8 polymerizes to a broader degree; and as indicated by the HPLC and MS/MS results, CP5 is more efficient for glycan production than CP8.

[0326] In *wzy* dependent polymerization pathways, it has been reported by Marolda, et al., that a specific enzyme (wzz or cld for chain length determinant) is responsible for determining the average number of RU polymerization steps performed (Marolda, C. L., L. D. Tatar, *et al.* (2006). "Interplay of the Wzx translocase and the corresponding polymerase and chain length regulator proteins in the translocation and periplasmic assembly of lipopolysaccharide o antigen." J Bacteriol 188(14): 5124-5135.). Wzz enzymes cause a specific repeat number averages, e.g. short, long and very long sugar polymers and are known to transfer their length specificity to exogenous polysaccharide pathways. The lengths and amounts of the CP8 glycolipids were analyzed in the production strain resulting in longer and lower amount of this sugar. To increase the amount of molecules and thereby the sugar transfer efficiency for protein glycosylation, a downregulation of the CP8 sugar length was performed using a specific Wzz enzyme.

[0327] To test the effect of a Wzz protein on the size and amounts of CP8 sugars on lipid, coexpression of Wzz from *E. coli wzz* O7 was performed from a separate plasmid (SEQ ID NO: 19). FIG. 11K presents the results of this test. Methanol extracts from *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* either expressing the chimeric CP8 cluster SEQ ID NO: 4 and a plasmid borne, IPTG inducible copy of wzzO7 (SEQ ID NO: 21, solid line), or an empty plasmid control (dashed line) were solid phase extracted on Sep-PAK cartridges and treated with mild acid to hydrolyse sugars from UndPP. 2AB labeled glycans were analyzed by normal phase HPLC. Alkali treatment of the CP8 sample showed that more than 85% of the area between 95 and 115' represents 7 or 8 RU polymers of CP8, indicating a wide variety of acetylation. These results also indicate that the chimeric CP8 cluster induced: a) an intensification in repeat numbers of the most abundant glycan from 7 to 8, and b) a higher overall intensity of fluorescent signal as judged from the area under the chromatogram.

[0328] Alkali treatment confirmed the acetylation of the shortened glycan as in FIG. 11I and 11J indicating that a recombinant polysaccharide's length can be regulated by a foreign Wzz enzyme. It is also possible to regulate the capsular sugar polymer length by an O-antigen derived Wzz enzyme. Furthermore, different promoters in front of the chimeric cluster when present on a plasmid cause different expression levels and different degrees of polymerization.

Example 5: Protein glycosylation with the CP5 and CP8 glycans and product characterization

[0329] Different variants of the chimeric cluster were tested for bioconjugate production. The chimeric O11/CP5 gene clusters (SEQ ID NO: 2 and 3), which contain different variants of *S. aureus* specificity regions in the O11 O-antigen cluster in place of *wbjA* and *wzy*, were expressed in the host strain *E. coli* W3110 *ΔwaaL ΔwecAwzzE::cat* in the presence of PglB (SEQ ID NO: 27?) and EPA (SEQ ID NO: 13). W3110 *ΔwaaL ΔwecAwzzE::cat* host cells expressed EPA with two glycosylation sites (from SEQ ID NO: 13) and PglB (SEQ ID NO: 27) from separate plasmids in addition to the pLAFR1 plasmid with the O11 O-antigen cluster where the *wbjA* and *wzy* genes were replaced with different *cap5* gene sets (and the *cat* cassette, SEQ ID NO: 2 and SEQ ID NO: 3).

[0330] The EPA protein is expressed containing: a) a N-terminal signal peptide sequence for export to the periplasm, b) two bacterial N-glycosylation consensus sequences engineered into the protein sequence (SEQ ID NO: 13) as set forth in Example 10 of WO 2009/104074, and c) a hexa histag for purification. The cells were grown in 5 L Erlenmeyer flasks in LB medium. An overnight culture was diluted to OD_{600nm}= 0.05. At OD_{600nm} around 0.5, PglB expression was induced by addition of 1 mM IPTG and EPA expression was induced by addition of arabinose (0.2 % final concentration). The cells were grown for 4 hours, induction was repeated and cells were grown for around additional 16 hours. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. EPA-CP5 bioconjugate without and with the *S. aureus* flippase gene *cap5K* (SEQ ID NO: 2 and 3) was eluted by 0.5M imidazole, and eluted peaks were pooled and analyzed by SDS PAGE and stained by Coomassie and silver (FIG. 12).

[0331] FIG. 12 presents the SDS PAGE results. The left panel shows the coomassie stain, and the right panel shows the silver stain. The numbers in the middle indicate the sizes of the molecular weight marker. The letters below the lanes indicate the genes that were present in the chimeric cluster expressed in the strains used for bioconjugate production. The host strain was *E. coli* W3110 *ΔwaaL ΔwecAwzzE::cat*. The results show protein signal at 70 kDa (electrophoretic mobility) most likely corresponding to unglycosylated EPA, and a ladder of bands above (100-170 kDa). The ladder likely corresponds to EPA protein glycosylated with the CP5 recombinant *S. aureus* glycan. In addition, the results indicate that including the flippase gene in the system increases the glycoprotein yield (middle and right lanes).

[0332] In a separate analysis, CP5-EPA bioconjugate was produced in *E. coli* W3110 *ΔwaaL ΔwecAwzzE::cat* by co-expression of the chimeric CP5 gene cluster (SEQ ID NO: 3), PglB (SEQ ID NO: 27) from plasmid pEXT21 and EPA (containing two glycosylation sites, SEQ ID NO: 13) from separate plasmids. To obtain a more controlled process for bioconjugate production, the cells were grown in a 2-L bioreactor to an $OD_{600\ nm}$ = 30 at 37°C, and expression of PglB and EPA was induced by the addition of 1 mM IPTG and 0.2% arabinose. The cells were grown for 18 h at 37°C under oxygen-limiting conditions. The cells were pelleted by centrifugation, washed and resuspended in 25% sucrose buffer at an $OD_{600\ nm}$ = 200, after 30 min. incubation at 4°C, the suspension was pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins present in the supernatant were loaded on a Ni^{2+} affinity chromatography. Glycosylated and unglycosylated EPA were eluted from the affinity column by 0.5 M imidazole and loaded on a SourceQ anionic exchange column. Glycosylated EPA was separated from unglycosylated EPA by applying a gradient of increasing concentration of NaCl.

[0333] As shown in FIG. 13A, the purified glycosylated EPA (CP5-EPA) was separated by SDS PAGE and stained by Coomassie (left lane) or transferred to nitrocellulose membranes and incubated with either anti CP5 antibodies (middle lane) or anti EPA antibodies (right lane). The purified bioconjugate was recognized by the EPA-specific antibodies (right lane), as well as the CP5-specific polyclonal antiserum (middle lane). The arrow indicates the position in the gel from where a piece was cut and used for trypsinization and analysis of glycopeptides by MALDI-MS/MS. FIG. 13B presents the MALDI-MS/MS of M/Z masses found for the glycosylation site in trypsinized peptide DNNNSTPTVISHR N-glycosidically linked to the O-acetylated RU mass (m/z=2088 ($[M+H]^+$)). MS/MS analysis of the m/z=2088 shows partial fragmentation of the sugar moiety as indicated. The inset illustrates the RU structure attached to the peptide derived from trypsinization of purified CP5-EPA from FIG. 13A. Sequential losses of ManNAcA (HexNAcA, 217 Da) and acetylated FucNAc (dHexNAc(OAc), 229 Da) support the expected glycan structure. FIG. 13C presents the MALDI-MS/MS of M/Z masses found for the glycosylation site in trypsinized peptide DQNR N-glycosidically linked to the O-acetylated RU mass (m/z=1165 ($[M+H]^+$)). MS/MS analysis of m/z=1165 shows the full Y-ion fragmentation ion series consistent with the CP5 RU structure. The inset illustrates the RU structure attached to the peptide derived from trypsinization of purified CP5-EPA from FIG. 13A. Sequential losses of ManNAcA (HexNAcA, 217 Da), acetylated FucNAc (dHexNAc(OAc), 229 Da), and FucNAc (dHexNAc, 187 Da) are shown, confirming the expected glycan structure on the peptide DQNR (m/z=532 Da ($[M+H^+]$)).

[0334] In FIG. 13D the CP8 bioconjugate in *E. coli* was produced using the same strategy as production of the CP5 bioconjugate. CP8-EPA bioconjugate was produced in *E. coli* by co-expression of the chimeric CP8 gene cluster (SEQ ID NO: 4), PglB (within the pEXT21 plasmid (SEQ ID NO: 27)), and EPA containing two glycosylation sites (SEQ ID NO: 13). Cells were grown in a bioreactor with a starting volume of 7 L in semi-defined medium containing glycerol, peptone and yeast extract as C-sources. Cells were grown at 37°C in batch or pulsed-batch mode to an $OD_{600\ nm}$ of 30, and expression of PglB and EPA was induced by the addition of 1 mM IPTG and 10% arabinose. After induction, cells were further cultivated in fed-batch mode for a period 15 hours under oxygen-limiting conditions. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+}

affinity chromatography. Glycosylated and unglycosylated EPA were eluted from the affinity column by 0.5 M imidazole and loaded on a SourceQ anionic exchange column. Glycosylated EPA was separated from unglycosylated EPA by applying a gradient of increasing concentration of NaCl.

[0335] As depicted in FIG. 13D, the purified protein was separated by SDS PAGE and stained by Coomassie (left lane) or transferred to nitrocellulose membranes and incubated with either anti CP8 antibodies (right lane) or anti EPA antibodies (middle lane).

[0336] Different strategies for further improving the glycosylation system were tested. In one strategy, to reduce the plasmid number in the production system to lower the burden of an additional antibiotic as well as maintaining an extra plasmid, the expression cassette for *pglB* was cloned into the plasmid containing the chimeric clusters for CP5 (SEQ ID NO: 17) and CP8 (SEQ ID NO: 18). The expression cassette is composed of the intergene region present between *galF* and *wbqA* of the *E. coli* O121 genome (for a promoter sequence), and the *pglB* sequence downstream of this. This expression cassette was cloned immediately downstream of the CP5 and CP8 chimeric clusters. We tested *E. coli* W3110 *ΔwaaL ΔwecAwzzECA::cat* containing the chimeric CP5 cluster (SEQ ID NO: 3) and pglB (SEQ ID NO: 27) on either separate plasmids or on the same plasmid (SEQ ID NO: 17). In addition, EPA (SEQ ID NO: 13) was expressed from a plasmid under the control of an arabinose inducible promoter. The cells were grown in 5 L Erlenmeyer flasks in LB medium at 37°C. An overnight culture was diluted to OD_{600nm}= 0.05. At OD_{600nm} around 0.5 PglB expression was induced by addition of 1mM IPTG and EPA expression was induced by addition of arabinose (0.2 % final concentration). The cells were grown for 4 hours, induction was repeated and cells were grown for around an additional 16 hours. The culture was pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. EPA-CP5 was eluted by 0.5M imidazole, and eluted peaks were pooled and analyzed by SDS PAGE and by Coomassie. FIG. 13E depicts the SDS PAGE results. Cells containing either 3 (left) or 2 plasmids (right lane) for glycoconjugate production are shown. The results show that glycolipid and conjugate production for CP5-EPA was maintained.

[0337] A further optimization of the system was the integration of the *wzz* (polymer length regulator) protein sequence in the plasmids used for protein glycosylation. Exemplified by the system producing CP8-EPA, *wzz* was integrated into the plasmid borne chimeric CP8 cluster (SEQ ID NO: 19) and downstream of the *epa* gene within the expression plasmid for the carrier protein (SEQ ID NO: 20). CP8-EPA bioconjugate was produced in *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat* comprising 2 plasmids: one plasmid contained in addition to the chimeric CP8 gene cluster a copy of the *wzz* O7 gene and a DNA cassette for the constitutive expression of the *pglB* gene (SEQ ID NO: 19); the second plasmid contained first the gene for expression and secretion of the detoxified EPA protein containing two glycosylation sites, and second a wzzO7 copy under the control of the same promoter (SEQ ID NO: 20). The resulting strain, *E. coli* W3110 *ΔwaaL ΔwecAwzzECA ΔrmlB-wecG::cat*, containing the mentioned plasmids was grown in a bioreactor with a starting volume of 7 L in semi-defined medium containing glycerol, peptone and yeast extract as C-sources. Cells were grown in batch or pulsed-batch mode to an $OD_{600\ nm}$ of 30, and expression of PglB and EPA was induced. After induction, cells were further cultivated in fed-batch mode for a period 15 hours under oxygen-limiting conditions and collected by centrifugation. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. Glycosylated and unglycosylated EPA were eluted from the affinity column by 0.5 M imidazole. Formation of glycoconjugate CP8-EPA is shown in FIG. 13F by Coomassie and western blot using anti his and anti CP8 antisera. FIG. 13F shows the results of SDS PAGE separation of the purified protein and analysis by Coomassie staining (left lane) or transferred to nitrocellulose membranes and probed with either anti histag antibodies (middle lane) or anti CP8 antibodies (right lane).

[0338] Characterization of the CP5-EPA glycoconjugate was further refined by various analytical methods. CovalX (Schlieren, Switzerland) performed High Mass MALDI analysis of a purified CP5-EPA sample produced using the 3 plasmid system as used in the analyses depicted in FIG. 13A in W3110 *ΔwaaL ΔwecAwzzECA::cat*. FIG. 14A depicts the High Mass MALDI results. A^+ and B^+ point towards mass protein species ($[M+H]^+$) corresponding to unglycosylated EPA and glycosylated EPA, respectively. Oligomeric forms may be present at higher molecular weight and signals in the low MW area are contaminants or degradation products. The results presented in FIG. 14A show that the above protein preparation contained a largely monomeric protein population which is 4 kDa larger than the EPA protein alone, indicative of a medium sugar length of 5.2 repeating units. This is in agreement with the sugar length of 5-7 of the major glycoconjugate form in the preparation as analyzed by SDS-PAGE, Coomassie brilliant blue staining and counting the repeating units in the major conjugate form (see FIGs. 7, 8, and 13A).

[0339] CP5-EPA was further characterized by size exclusion chromatography (SEC-HPLC). We used the 3 plasmid system in W3110 *ΔwaaL ΔwecAwzzECA::cat* as used in the analyses depicted in FIG. 13A. The sample was purified by anionic exchange chromatography to remove unglycosylated EPA. Analysis was performed on a Supelco TSK G2000SWXL column. FIG. 14B shows the results of the SEC-HPLC analysis of the purified CP5-EPA sample. The UV trace measured at 280 nm is shown. The thick solid line derives from analyzing 3.25 μg purified CP5-EPA, the thin line was obtained from 5 μg purified, unglycosylated EPA. A major, homogenous peak at 11.5 minutes of elution is shown, whereas unglycosylated EPA eluted at 12.9 minutes (FIG. 14B). Calculation of the hydrodynamic radii of the two molecules resulted in a size of 42 kDa for unglycosylated EPA and 166 kDa for glycosylated EPA. This indicates that glycosylated EPA appears as an elongated, monomeric protein in solution as expected due to the linear structure of the glycan.

[0340] Our analyses therefore confirmed that the CP5-EPA bioconjugate consists of the EPA protein and the correct, O-acetylated glycan structure. Based on these results, it could also be predicted that the CP8-EPA bioconjugate consisted of the EPA protein and the correct, O-acetylated glycan structure.

Example 6: *S. aureus* protein glycosylation and product characterization

[0341] To prove the versatility of the "in vivo" glycosylation to generate glycoconjugate vaccine candidates several carrier proteins were used as substrate to be glycosylated with CP5. To further increase the immune response of the bioconjugate vaccine against *S. aureus,* the carrier protein EPA is exchanged by AcrA form *C. jejuni* and two proteins from *S. aureus*: Hla and ClfA. To be used as carrier proteins Hla and ClfA were modify by the insertion of the bacterial

N-glycosylation sites. The process was performed as described in WO 2006/119987 generating four versions for Hla H35L: SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 16 and three for ClfA: SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12.

[0342] For glycosylation of Hla H35L site 130 *E. coli* cells (W3110 Δ*waaL* Δ*wecAwzzE* Δ*rmlB-wecG*) comprising two expression plasmids: one for Hla H35L production (SEQ ID NO: 16), in which expression of the Hla H35L containing the N-terminal signal peptide for periplasmic secretion, one *N*-glycosylation site and a hexa HIS-tag for purification is under control of the ParaBAD promoter, and secondly one for expression of the CP5 chimeric cluster and pglB (SEQ ID NO.: 17) were used. This system corresponds to the beforehand optimized 2 plasmids expression system of CP5-EPA with an exchanged protein carrier expression plasmid. Cells were grown in a 12-L bioreactor in rich medium to an $OD_{600\ nm} = 30$, expression of Hla was induced by the addition 0.2% arabinose. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins in the supernatant were loaded on a Ni^{2+} affinity chromatography. Glycosylated (CP5-Hla) and unglycosylated Hla were eluted from the affinity column by 0.5 M imidazole and loaded on an anionic exchange chromatography Proteins were eluted with a linear gradient from 0 to 0.7 M NaCl to separate CP5-Hla from Hla. The resulting protein was separated by SDS PAGE and stained by Coomassie, or transferred to nitrocellulose membranes and probed with either anti His, anti Hla, or anti CP5 antisera, as indicated (FIG. 14C). The results in FIG. 14C show the formation of glycoconjugate (CP5-Hla) by coomassie (left lane) and western blot using anti His (middle left lane) and anti Hla (middle right) and anti CP5 (right) antisera.

[0343] The identity of Hla H35L with an engineered glycosylation site 130 was confirmed by in-gel trypsinization and MALDI-MS/MS.

[0344] To further show that the carrier protein is exchangeable for glycosylation by CP5 and CP8, *C. jejuni* AcrA protein was used as a glycosylation acceptor (see FIG. 14D). Using the 3 plasmid system (SEQ ID NO: 3, SEQ ID NO: 15, and SEQ ID NO: 27), the production strain for this conjugate was W3110 Δ*waaL* habouring the CP5 chimeric cluster (SEQ ID NO: 3), the

PglB protein induced by IPTG (SEQ ID NO: 27) and the AcrA (SEQ ID NO: 15) under arabinose induction on separate plasmids. Cells were grown in a bioreactor with a starting volume of 7 L in semi-defined medium containing glycerol, peptone and yeast extract as C-sources. Cells were grown in batch or pulsed-batch mode to an $OD_{600\ nm}$ of 30, and expression of PglB and AcrA was induced by the addition of 1 mM IPTG and 10% arabinose. After induction, cells were further cultivated in fed-batch mode for a period 15 hours under oxygen-limiting conditions and collected by centrifugation. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. CP5-AcrA glycoproteins were eluted from the affinity column by 0.5 M imidazole. The purified protein was separated by SDS PAGE and stained by Coomassie, or transferred to nitrocellulose membranes and probed with either anti AcrA, or anti CP5 antisera, as indicated in FIG. 14D.

[0345] The insertion of the bacterial N-glycosylation sites in ClfA was performed as described in WO 2006/119987, generating SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12. The carrier proteins were expressed in *E. coli* cells from arabinose inducible promoters. The genes were designed to produce a N-terminal signal peptide for periplasmic secretion, several *N*-glycosylation sites and a hexa HIS-tag for purification. Purification was started from periplasmic extracts of *E. coli* cells.

[0346] For glycosylation of ClfA 327 the beforehand optimized expression systems of CP5-EPA was employed. Using the 2 plasmid system (SEQ ID NO: 17 and SEQ ID NO: 11), *E. coli* cells (W3110 Δ*wecAwzzE* Δ*rmlB-wecG* Δ*waaL*) comprising the CP5 chimeric cluster and *pglB* (constitutive expression cassette) as well as the expression plasmid for ClfA 327(under control of the ParaBAD promoter) were grown in 1 L Erlenmeyer flasks in LB medium. An overnight culture was diluted to $OD_{600\ nm}$= 0.05. At OD_{600nm} around 0.5, ClfA expression was induced by addition of arabinose (0.2 % final concentration). The cells were grown for 20 hours. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. ClfA-CP5 was eluted by 0.5M imidazole, was separated by SDS PAGE and stained by Coomassie, or transferred to nitrocellulose membranes and probed with either anti His, or anti CP5 antisera. FIG. 14E shows the results obtained using the ClfA variant with the glycosylation site inserted around amino acid position 327 of the protein (SEQ ID NO: 11). They show the formation of ClfA by Coomassie staining and anti His western blot, and glycoconjugate (CP5-ClfA) by western blot using anti CP5 antisera.

Example 7: Activity of CP5-EPA as glycoconjugate vaccine

[0347] W3110 *ΔwaaL ΔwecAwzzECA::cat* cells comprising CP5 chimeric cluster (SEQ ID NO: 3) with cap5K inside, the PglB protein (SEQ ID NO: 27) and EPA with signal 2 glycosylation sites on pEC415 (SEQ ID NO: 13) were grown in 1 L Erlenmeyer flasks in LB medium. An overnight culture was diluted to OD_{600nm}= 0.05. At OD_{600nm} around 0.5, EPA and PglB expression was induced by addition of arabinose (0.2 % final concentration) and 1mM IPTG, respectively. The cells were grown for 20 hours. Cells were pelleted by centrifugation; the cells were washed and suspended in 0.2 vol sucrose buffer, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. Glycosylated and unglycosylated EPA were eluted from the affinity column by 0.5 M imidazole and loaded on a SourceQ anionic exchange column. Glycosylated EPA was separated from unglycosylated EPA by applying a gradient of increasing concentration of NaCl. Eluted protein amounts were determined by the BCA assay and based on the size of the bands obtained on SDS PAGE stained by Coomasie the theoretical mass of the sugar was calculated. Together with the protein determination, the amount of polysaccharide antigen was estimated in the preparation. This estimated quantification was confirmed by high mass maldi MS method (see FIG. 14A).

[0348] To measure the immunogenicity of CP5-EPA in living animals, 1 μg of the purified glycoconjugate was injected into mice by the IP (intra peritoneal) route in the presence of Aluminium hydroxide as adjuvant on days 1 (first injection), 21 (second injection), and 56 (third injection). After 35 and 61 days, which were two weeks after the second and third injections, respectively, the IgG response was measured by ELISA using a poly-L-lysine modified CP5 for coating (Gray, B.M. 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. 28:187-192). Blood from mice immunized with CP5-bioconjugate was analyzed for specific IgG antibodies against CP5 capsular polysaccharide. FIG. 15A presents the IgG titers raised by CP5-EPA in mice. ELISA plates were coated with poly-L-lysine modified CP5, IgG response in mice immunized twice (second bar (empty) at each dilution) or three times (first bar (forward diagonals) at each dilution) with CP5-EPA was measured in triplicates. The signals obtained with the preimmune sera as control are indicated by the third bar (backward diagonals) at each dilution. The mice IgG response was measured with alkaline phosphatase-conjugated protein G. As shown in FIG. 15A, the CP5-EPA bioconjugate elicited a serum antibody titer of 6.4×10^3. The results presented in FIG. 15A show that CP5-EPA raises CP5 specific antibodies in mice. This experiment shows that the bioconjugate produced in *E. coli* is immunogenic in mice.

[0349] A similar experiment was performed in rabbits as the host organism. CP5-EPA (15 μg CP5) was injected into rabbits intra-dermal in the presence of Freund´s complete adjuvant on day 1 and subcutaneously in the presence of Freund´s incomplete adjuvant on days 20, 30 and 40. After 61 days, the IgG response was measured by ELISA using a poly-L-lysine modified CP5 for coating (Gray, B.M. 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. 28:187-192). FIG. 15B presents IgG titers raised by CP5-EPA in rabbits. The results presented in FIG. 15B show that CP5-EPA raises CP5 specific antibodies in rabbits. Immune response to CP5-EPA bioconjugate is the second bar (forward diagonals) at each dilution. Control sera include CP5-specific absorbed sera raised to killed *S. aureus* (WC extracts, first bar (dots) at each dilution) and preimmune serum (third bar (empty) at each dilution). Serum from rabbits immunized with various antigens was analyzed for specific antibodies to purified CP5. Plates were coated with poly-L-lysine modified CP5. The signals obtained with the preimmune sera as control are indicated by the third bar (backward diagonals) at each dilution. The rabbit IgG response was measured with alkaline phosphatase-conjugated protein G in triplicates. The CP5-EPA bioconjugate elicited a titer of 1×10^6, which was 4 times higher than the titer of control sera (prepared by immunization with whole killed *S. aureus* and then absorbed with Wood 46 and a trypsinized isogenic acapsular mutant, so that the antiserum was rendered CP5-specific). This experiment shows that the bioconjugate was able to elicit a high-titered CP5-specific IgG response.

Example 8: Functional activities of CP5 antibodies

In vitro activity

[0350] The rabbit polyclonal antiserum raised as described in Example 7 was purified by Protein A affinity column to enrich for IgG specific antibodies. IgG from rabbits immunized with *S. aureus* bioconjugate CP5-EPA was tested for functional activity in a classic *in vitro* opsonophagocytic killing assay (Thakker, M., J.-S. Park, V. Carey, and J. C. Lee. 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun 66:5183-5189). *S. aureus* was cultivated for 24 h on Columbia agar + 2% NaCl. The bacteria were suspended in minimal essential medium + 1% BSA (MEM-BSA). PMNs (polymorphonuclear neutrophils) were isolated from fresh human blood, washed, counted, and suspended in MEM-BSA. The purified IgG preparations from rabbits immunized with either *S. aureus* CP5-EPA or as control purified IgG preparations from rabbits immunized with Shigella O1-EPA that has been purified as described in WO 2009/104074 were added to the assay in serial 10-fold dilutions prepared in MEM-BSA. Guinea pig serum (Pel-Freez) was used as a C' source. Each assay (0.5 ml total volume) contained ~5 x 10^6 PMNs, 1 x 10^6 CFU *S. aureus*, 0.5% to 1% guinea pig serum, and varying concentrations of IgG, ranging from 140 µg/ml to 1 µg/ml. Control samples contained 1) *S. aureus* incubated with C' and PMNs, but no antibody; 2) *S. aureus* incubated with IgG and C', but no PMNs; and 3) *S. aureus* alone. The samples were rotated end-over-end (12 rpm) for 2 h at 37°C. Sample dilutions were vortexed in sterile water, and bacterial killing was estimated by plating the diluted samples in duplicate on TSA. The percent killing was defined as the reduction in CFU/ml after 2 h compared with that at time zero.

[0351] In the first set of experiments, the opsonophagocytic killing of the methicillin-sensitive *S. aureus* (MSSA) strain Reynolds, the prototype CP5 isolate, was tested, and the results are shown in FIG. 16A. Opsonic activity of antibodies to CP5-EPA raised in rabbit was tested against the *S. aureus* serotype 5 strain Reynolds. CP5-EPA antibodies were opsonic down to a concentration of 1.4 µg/ml, whereas O1-EPA antibodies showed little opsonic activity at 140 µg/ml. A positive control serum raised against *S. aureus* whole cell extracts (obtained from J. C.

Lee at the Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA) showed similar activity as the anti CP5-EPA serum (WC antiserum 1%).

[0352] As shown in FIG. 16A, between 65-75% of *S. aureus* Reynolds was killed by PMNs when incubated with antibodies to CP5-EPA and 1% guinea pig serum with complement activity. The antiserum was used at a final 1% in the assay, and 89% of the *S. aureus* inoculum was killed under these conditions. Little killing was observed when *S. aureus* was opsonized by C' alone (1% guinea pig serum) or antibodies and C' with no PMNs. The data shown are the means of 2 to 5 experiments. All samples graphed included guinea pig serum C', and no killing was observed in the absence of C'. Neither antibodies alone nor complement alone were opsonic, and this feature is characteristic of encapsulated bacterial pathogens. In contrast, antibodies elicited by the control vaccine (Shigella O1 antigen coupled to EPA) did not show opsonic activity, even in the presence of C'. As a positive control in this assay, we also tested CP5-specific rabbit antiserum (obtained from J. C. Lee at the Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). These data show that antibodies raised to the CP5-EPA bioconjugate showed opsonic activity against encapsulated *S. aureus* that is comparable to CP5 antibodies with documented opsonic activity (Thakker, M., J.-S. Park, V. Carey, and J. C. Lee. 1998. *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun 66:5183-5189).

[0353] The opsonic activity of antibodies to CP5-EPA tested against the MRSA strain USA100 of CP5-EPA. FIG. 16B presents the results of the opsonic activity of IgG and C' tested against *S. aureus* strain USA100, a CP5+ isolate, and is called NRS382. The data shown are the means of 2 to 5 experiments. All samples graphed included guinea pig serum C', and no killing was observed in the absence of C'. As shown in FIG. 16B, ~60% of the USA100 inoculum was killed by PMNs incubated with 0.5% guinea pig complement and concentrations of CP5-EPA IgG ranging from 100 to 1 μg/ml. Minimal killing was observed in the absence of PMNs or when IgG was omitted from the assay. No killing was achieved when IgG raised to the O1-EPA conjugate vaccine was added to PMNs + C' (the bacteria multiplied in this sample). Little killing was observed when *S. aureus* was opsonized by C' alone or antibodies and C' with no PMNs. Thus, CP5-EPA antibodies were opsonic at concentrations ranging from 100 to 1 μg/ml, whereas O1-EPA antibodies showed little opsonic activity at 100 μg/ml. This experiment shows that CP5-EPA antibodies display opsonic activity against both MSSA and MRSA strains.

In vivo activity

[0354] To determine whether the opsonic activity of IgG raised to the bioconjugate CP5-EPA vaccine would predict protection in a mouse model of staphylococcal infection, passive immunization experiments were performed. In the initial studies, Swiss-Webster male mice (~6 wks of age) were injected IV (tail vein) with 1.4 to 2 mg IgG from rabbits immunized with CP5-EPA or Shigella O1-EPA. After 24 h, the mice were challenged by the intra-peritoneal (IP) route with ~3.6 x 10^7 CFU *S. aureus* Reynolds. Bacteremia levels were measured 2 h after challenge to assess antibody-mediated clearance of the bacteremia. The lower limit of detection by culture was 5 CFU/ml blood. FIG. 17A show the resulting bacteremia levels. Each dot represents a quantitative blood culture performed by tail vein puncture on an individual mouse 2 h after bacterial inoculation. Horizontal lines represent median CFU/ml values. Empty circles are blood samples from mice that obtained anti CP5-EPA antibodies, black filled circles are samples from animals that got a control antibody preparation which was raised against EPA conjugated to a different glycan (*S. dysenteriae* O1). The results of FIG. 17A show that mice given CP5 antibodies showed a significant (P = 0.0006 by Mann-Whitney analysis) reduction in bacteremia levels compared to mice given the O1-specific antibodies. In fact, the reduction in CFU/ml blood was 98% in mice passively immunized with the CP5-EPA vs. mice given O1-EPA IgG.

[0355] In subsequent passive immunization experiments, mice were challenged IP with a lower inoculum (~5.5 x 10^6 CFU/mouse) of *S. aureus* Reynolds. Passive immunization with CP5-EPA antibodies was tested in mice challenged IP with 5-6 x 10^6 CFU *S. aureus* Reynolds. Mice were injected intravenously (IV) with 2 mg CP5-EPA IgG or O1-EPA IgG 24 h before bacterial challenge. FIG. 17B shows the resulting bacteremia levels. Each dot represents a quantitative blood culture performed by tail vein puncture on an individual mouse 2 h after bacterial inoculation. Horizontal lines represent median CFU/ml values. Empty circles are blood samples from mice that obtained anti CP5-EPA antibodies, black filled circles are samples are from animal that got a control antibody preparation which was raised against EPA conjugated to a different glycan (*S. dysenteriae* O1). As shown in FIG. 17B, mice given 2 mg CP5-EPA IgG had significantly (P <0.0001 by Mann-Whitney analysis) lower bacteremia levels than animals given 2 mg of O1-EPA IgG. In fact, 6 of 7 mice passively immunized with CP5-EPA antibodies had sterile blood cultures (lower limit of detection 6 to 30 CFU/ml blood, depending on the blood volume collected and plated from each mouse). The reduction in bacteremia levels attributable to CP5 antibodies was 98%, compared to control mice given O1-EPA IgG.

[0356] To determine whether protection against bacteremia could be conferred by a lower level of IgG, a subsequent experiment was performed wherein mice were passively immunized by the IV route with 300 µg CP5-EPA or O1-EPA IgG. After 24 h, the mice were inoculated IP with 6 x 10^6 CFU *S. aureus* Reynolds. The lower limit of detection by culture was 13-67 CFU/ml blood. FIG. 17B shows the resulting bacteremia levels. Each dot represents a quantitative blood culture performed by tail vein puncture on an individual mouse 2 h after bacterial inoculation. Horizontal lines represent median CFU/ml values. Empty circles are blood samples from mice that obtained anti CP5-EPA antibodies, black filled circles are samples are from animal that got a control antibody preparation which was raised against EPA conjugated to a different glycan (*S. dysenteriae* O1). As in FIG. 17B, the results of FIG. 17C show CP5 antibody-mediated protection against bacteremia was achieved with this lower antibody dose. A 98% reduction in bacteremia levels was achieved by antibodies elicited by the CP5 bioconjugate vaccine, and 8 of 9 mice had sterile blood cultures compared to 0 of 8 mice given *Shigella* O1-EPA antibodies.

Example 9: Active immunization in mice

[0357] To show that vaccination of mice with the bioconjugate CP5-EPA mediates protection against bacterial challenge as in passive immunization assay, active immunization studies were performed.

[0358] CP5-EPA bioconjugate was produced in *E. coli* W3110 *ΔwaaL ΔwecAwzzE::cat* by co-expression of the chimeric CP5 gene cluster (SEQ ID NO: 3), PglB (SEQ ID NO: 27) from plasmid pEXT21 and EPA (containing two glycosylation sites, SEQ ID NO: 13) from separate plasmids. Cells were grown in a bioreactor with a starting volume of 7 L in semi-defined medium containing glycerol, peptone and yeast extract as C-sources. Cells were grown in batch or pulsed-batch mode to an $OD_{600\ nm}$ of 30, and expression of PglB and EPA was induced by the addition of 1 mM IPTG and 10% arabinose. After induction, cells were further cultivated in fed-batch mode for a period 15 hours under oxygen-limiting conditions and collected by centrifugation. The cells were washed and resuspended in 25% sucrose buffer at an $OD_{600\ nm} = 200$, pelleted, and lysed by osmotic shock. The spheroplasts were pelleted by centrifugation, and the periplasmic proteins were loaded on a Ni^{2+} affinity chromatography. Glycosylated and unglycosylated EPA were eluted from the affinity column by 0.5 M imidazole and loaded on a SourceQ anionic exchange column. Glycosylated EPA was separated from unglycosylated EPA by applying a gradient of increasing concentration of NaCl.

[0359] CP5-EPA is intended to be used as a conjugate vaccine to protect against CP5 *S. aureus* strains. To test whether such active immunization is functional, we immunized different groups of female Swiss Webster mice with three different doses of CP5-EPA and analyzed the immunization using a bacteremia model. Three doses were subcutaneously injected at days 0, 14 and 28. Mice were intra-peritoneally challenged at day 42 with *S. aureus* strain JL278, as shown in FIG. 18. Five groups of mice were immunized with three different doses of CP5-EPA as indicated below the x-axis (dotted circles; empty circles; and backward diagonals in circles). Two control groups received either adjuvants (forward diagonals in circles) or PBS (black filled circles) alone. Each dot represents a blood sample from a single mouse. The lowest dose of vaccine (0.2 μg) induced protection in all mice from the group. Two hours after challenge blood samples were analyzed for cfu formation and anti CP5 antibodies by ELISA using a poly-L-lysine modified CP5 for coating (Gray *et al.* (1979)). In all groups immunized with CP5-EPA, a mean reduction of cfu in blood was observed. However, only in the group which received the lowest dose of vaccine, there was a general protection from bacteremia in all five mice. Analysis of blood for anti CP5 antibodies resulted in a positive correlation of protection and mean ELISA titers in the different mouse groups. The results presented in FIG. 18 indicate that the antibodies induced the protection from bacteremia in immunized mice.

[0360] These studies indicate that the CP5-EPA bioconjugate vaccine induced antibodies that opsonized *S. aureus* for phagocytic killing by human PMNs and protected mice against bacteremia in positive and active immunization studies. These data provide strong evidence that the presented bioconjugate will protect against disease provoked by multiple *S. aureus* strains.

[0361] While this invention has been particularly shown and described with references to embodiments thereof, it will be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the scope of the invention encompassed by the claims.

CLAIMS

1. A *Staphylococcus aureus* vaccine comprising:

a protein comprising an inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline;

at least one *Staphylococcus aureus* polysaccharide linked to said consensus sequence through an N-glycosidic linkage; and a pharmaceutically acceptable carrier or adjuvant.

2. The *S. aureus* vaccine of claim 1, wherein said at least one *S. aureus* polysaccharide comprises capsular polysaccharide 5.

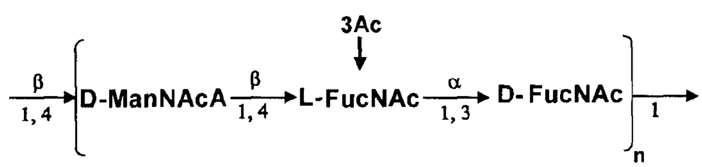
3. The *S. aureus* vaccine of claim 1, wherein said at least one *S. aureus* polysaccharide comprises capsular polysaccharide 8.

4. The *S. aureus* vaccine of claim 1, wherein said protein is *Pseudomonas aeruginosa* Exotoxin.

5. The *S. aureus* vaccine of claim 1, wherein said protein is *S. aureus* alpha hemolysin.

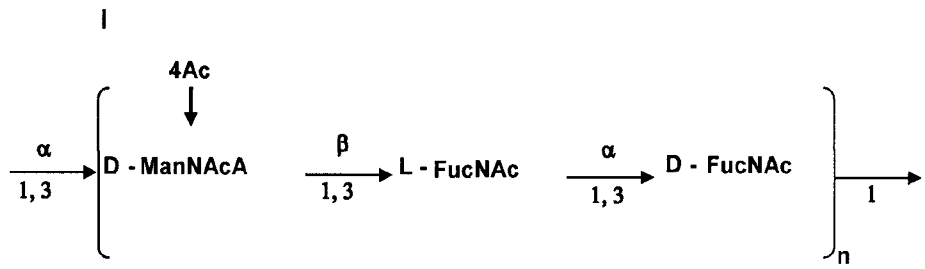
6. The *S. aureus* vaccine of claim 1, wherein said protein is *S. aureus* clumping factor A.

7. The *S. aureus* vaccine of claim 1, wherein said at least one *S. aureus* polysaccharide comprises the following structure:



wherein n is a number equal to or greater than 3.

8. The *S. aureus* vaccine of claim 1, wherein said at least one *S. aureus* polysaccharide comprises the following structure:



wherein n is a number equal to or greater than 3.

9. The *S. aureus* vaccine of claim 1, comprising two or more of said inserted consensus sequences and two or more of said *S. aureus* polysaccharides.

10. The *S. aureus* vaccine of claim 9, wherein said *S. aureus* polysaccharides comprise polysaccharides which target different *S. aureus* strains.

11. The *S. aureus* vaccine of claim 10, wherein said *S. aureus* polysaccharides comprise capsular polysaccharide 5 and capsular polysaccharide 8.

12. A recombinant *N*-glycosylated protein comprising:

a EPA, ClfA, Hla or AcrA protein comprising at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and

at least one *Staphylococcus aureus* polysaccharide N-linked to said consensus sequence.

13. The recombinant *N*-glycosylated protein of claim 12, wherein said at least one *S. aureus* polysaccharide comprises capsular polysaccharide 5.

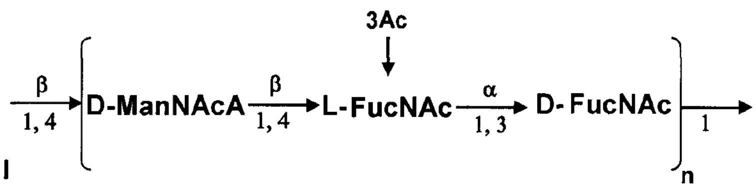
14. The recombinant *N*-glycosylated protein of claim 12, wherein said at least one *S. aureus* polysaccharide comprises capsular polysaccharide 8.

15. The recombinant *N*-glycosylated protein of claim 12, wherein said protein is *P. aeruginosa* Exotoxin.

16. The recombinant *N*-glycosylated protein of claim 12, wherein said protein is *S. aureus* alpha hemolysin.

17. The recombinant *N*-glycosylated protein of claim 12, wherein said protein is *S. aureus* clumping factor A.

18. The recombinant *N*-glycosylated protein of claim 12, wherein said at least one *S. aureus* polysaccharide comprises the following structure:

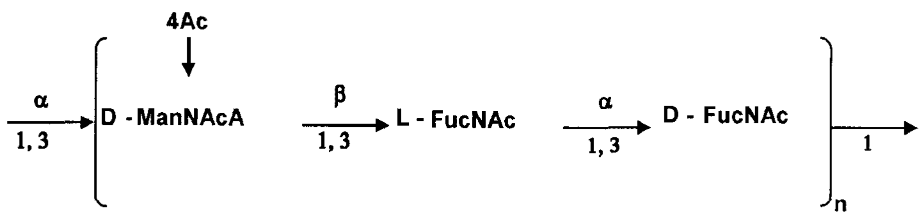


I

wherein n is a number equal to or greater than 3.

19. The recombinant *N*-glycosylated protein of claim 12, wherein said at least one *S. aureus* polysaccharide comprises the following structure:

I



wherein n is a number equal to or greater than 3.

20. The recombinant *N*-glycosylated protein of claim 12, comprising two or more of said inserted consensus sequences and two or more of said *S. aureus* polysaccharides.

21. The recombinant N-glycosylated protein of claim 20, wherein said *S. aureus* polysaccharides comprise polysaccharides which target different *S. aureus* strains.

22. The recombinant N-glycosylated protein of claim 21, wherein said *S. aureus* polysaccharides comprise capsular polysaccharide 5 and capsular polysaccharide 8.

23. An *Escherichia coli* Gram-negative host prokaryotic organism comprising:

a nucleotide sequence encoding at least one glycosyltransferase from a Gram-positive bacterium which is *Staphylococcus aureus*;

a nucleotide sequence encoding at least one glycosyltransferase from a Gram-negative bacterium which is *Pseudomonas aeruginosa*;

a nucleotide sequence encoding a EPA, ClfA, Hla or AcrA protein which comprises at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and

a nucleotide sequence encoding a PglB oligosaccharyl transferase.

24. The host prokaryotic organism of claim 23, wherein said *S. aureus* is a capsular polysaccharide 5 strain.

25. The host prokaryotic organism of claim 23, wherein said *S. aureus* is a capsular polysaccharide 8 strain.

26. The host prokaryotic organism of claim 23, wherein said protein is *P. aeruginosa* Exotoxin.

27. The host prokaryotic organism of claim 23, wherein said protein is *S. aureus* alpha hemolysin.

28. The host prokaryotic organism of claim 23, wherein said protein is *S. aureus* clumping factor A.

29. A method of modifying a bacterium of a first Gram-negative species which is *Escherichia coli* comprising:

selecting a Gram-positive bacterium which is *Staphylococcus aureus* as a target;

identifying a first repeating unit of a polysaccharide produced by said Gram-positive bacterium which is *Staphylococcus aureus* comprising at least three monomers;

identifying a polysaccharide produced by a bacterium of a second Gram-negative species which is *Pseudomonas aeruginosa* comprising a second repeating unit comprising at least two of the same monomers as said first repeating unit;

inserting into said bacterium of a first Gram-negative species which is Escherichia coli one or more nucleotide sequences encoding glycosyltransferases that assemble a trisaccharide containing:

a) said second repeating unit; and

b) a monomer of said first repeating unit not present in said second repeating unit;

inserting a nucleotide sequence encoding a EPA, ClfA, Hla or AcrA protein which comprises at least one inserted consensus sequence D/E-X-N-Z-S/T, wherein X and Z may be any natural amino acid except proline; and

inserting a nucleotide sequence encoding a PglB oligosaccharyl transferase.

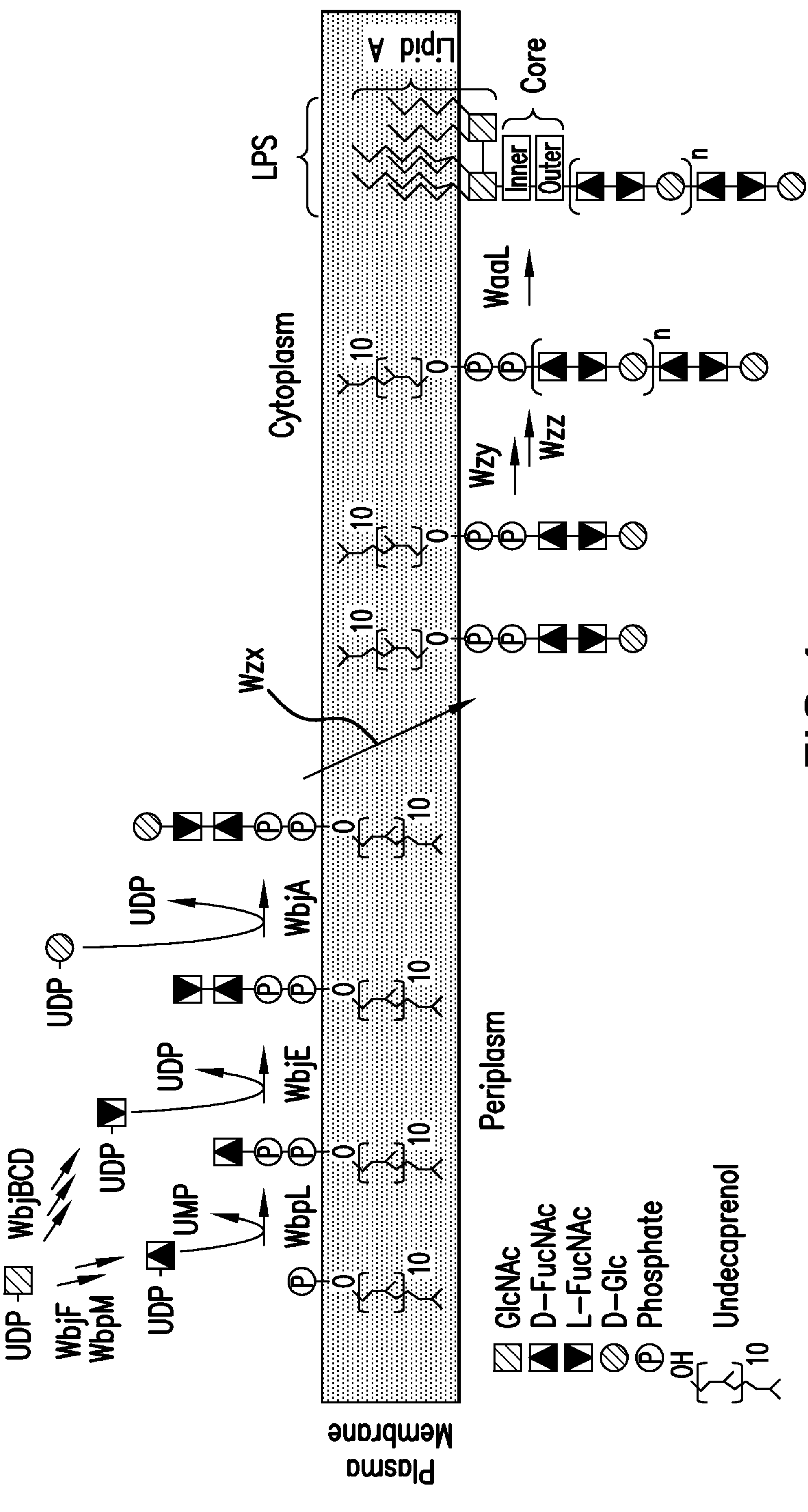

LPS
Lipid A
Core
Inner
Outer
n
Cytoplasm
WaaL
Wzy
Wzz
Wzx
Periplasm
Plasma Membrane
UDP
UMP
WbjA
WbjE
WbpL
WbjBCD
WbjF
WbpM
10
GlcNAc
D-FucNAc
L-FucNAc
D-Glc
Phosphate
Undecaprenol

FIG.1

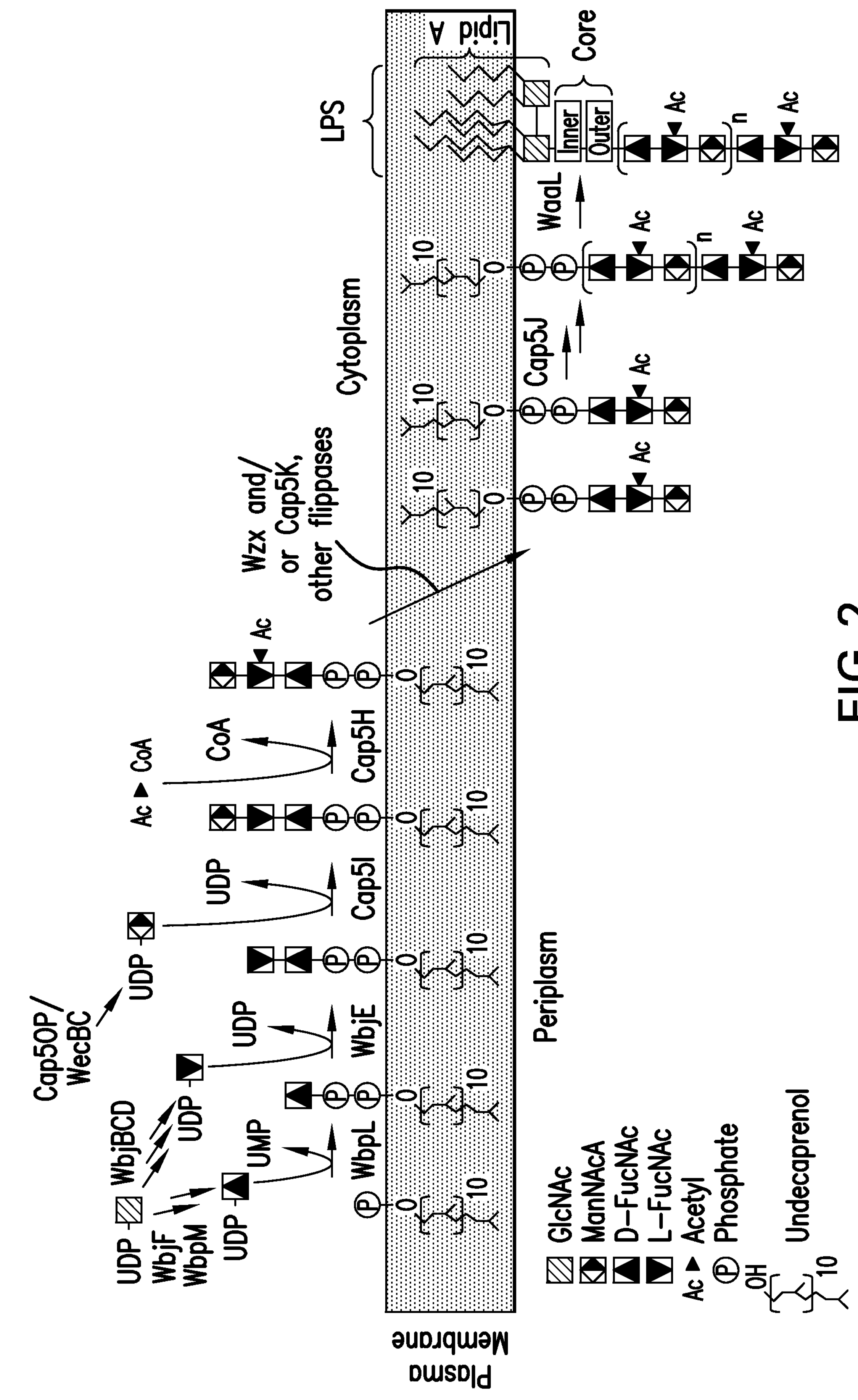

LPS
Lipid A
Core
Inner
Outer
Ac
n
WaaL
Cytoplasm
Cap5J
Wzx and/
or Cap5K,
other flippases
10
0
P
Ac ▶ CoA
CoA
Cap5H
UDP
Cap5I
Cap5OP/
WecBC
UDP
WbjE
UDP
WbjBCD
UMP
WbpL
WbjF
WbpM
Periplasm
Plasma
Membrane
GlcNAc
ManNAcA
D–FucNAc
L–FucNAc
Acetyl
Phosphate
OH
Undecaprenol

FIG.2

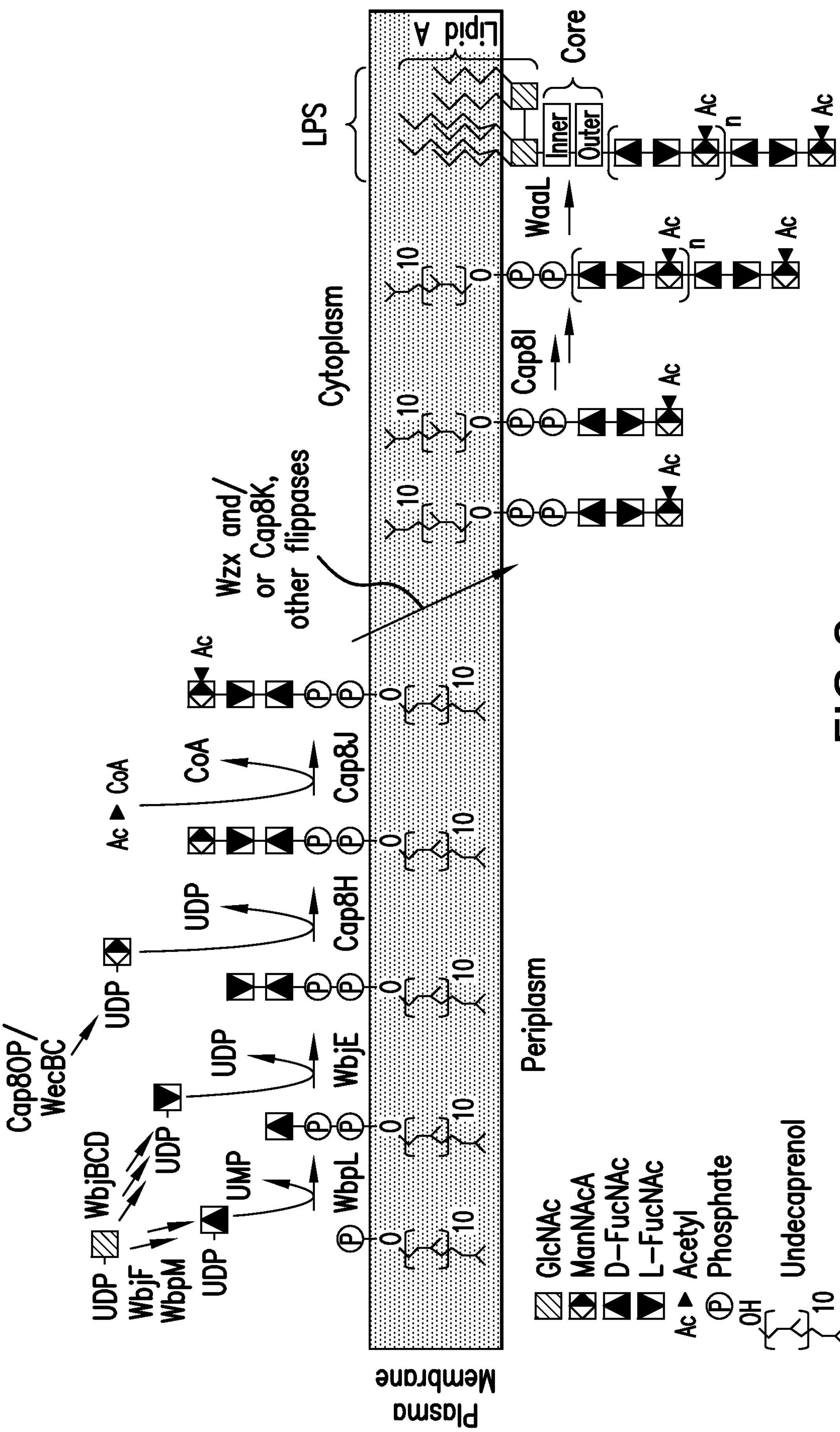

LPS
Lipid A
Core
Inner
Outer
Cytoplasm
Periplasm
Plasma Membrane
WaaL
Cap8I
Wzx and/
or Cap8K,
other flippases
Cap8J
CoA
Ac ▶ CoA
Cap8H
UDP
WbjE
Cap8OP/
WecBC
WbjBCD
WbjF
WbpM
WbpL
UMP
GlcNAc
ManNAcA
D-FucNAc
L-FucNAc
Acetyl
Phosphate
Undecaprenol

FIG.3

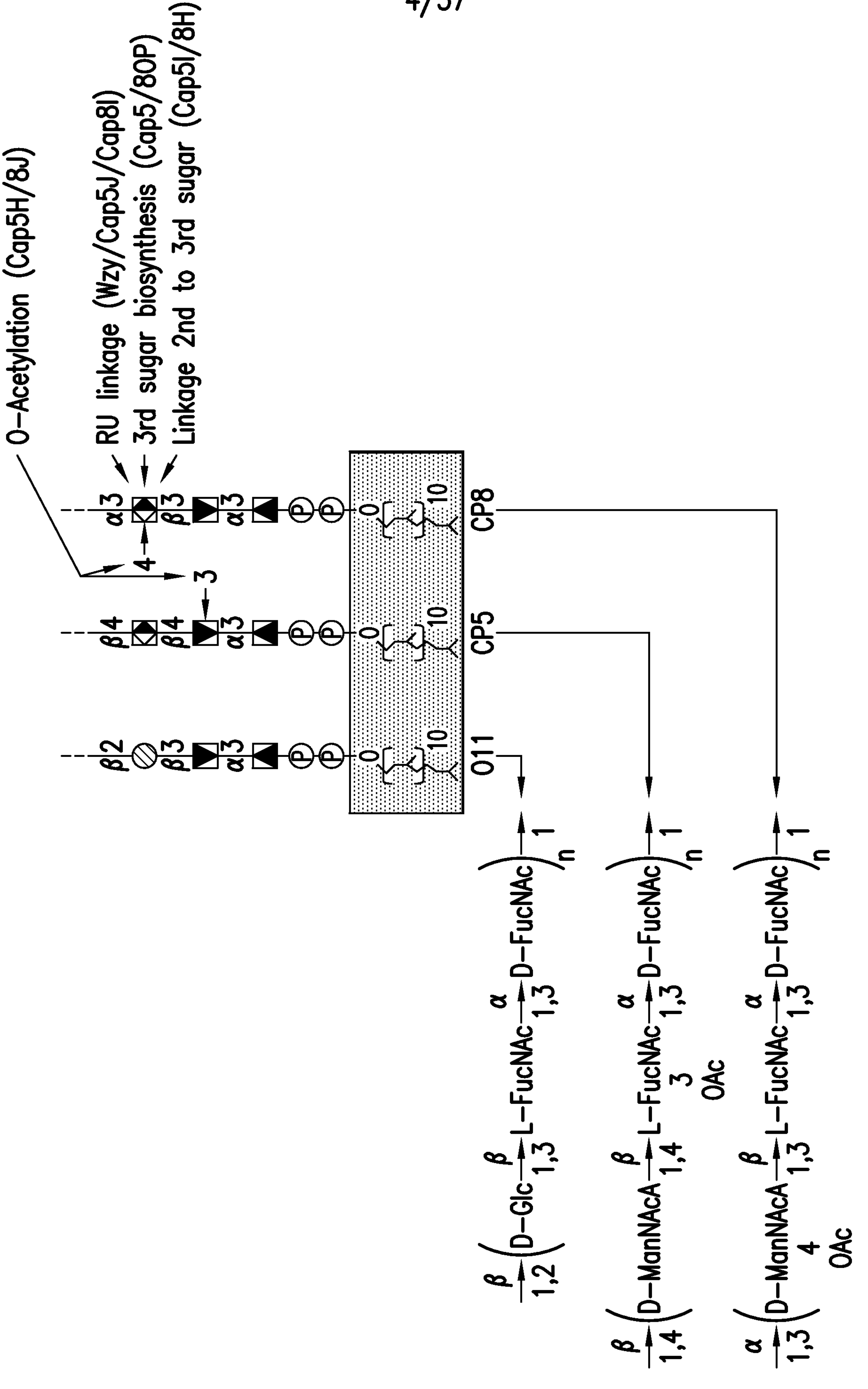
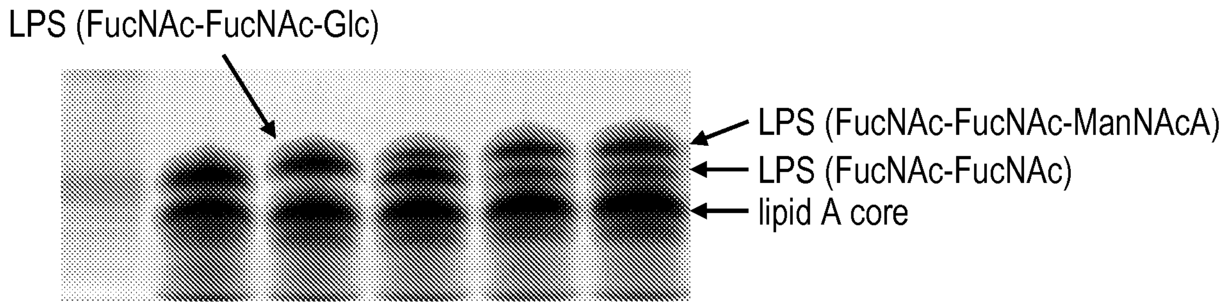
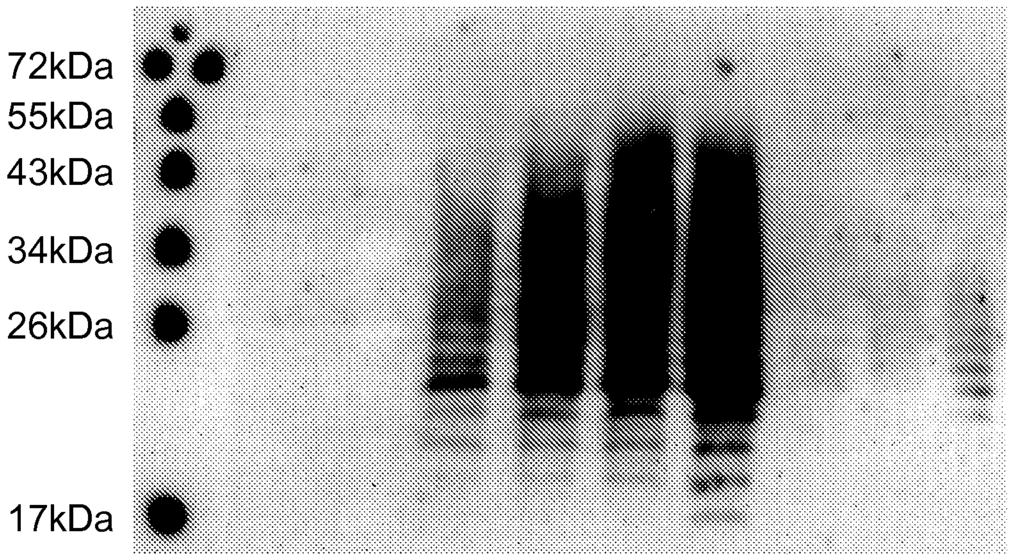

O-Acetylation (Cap5H/8J)
RU linkage (Wzy/Cap5J/Cap8I)
3rd sugar biosynthesis (Cap5/8OP)
Linkage 2nd to 3rd sugar (Cap5I/8H)
β2
β3
α3
β4
β4
α3
α3
β3
α3
P
P
O
10
O11
CP5
CP8
4
3
β 1,2 (D-Glc β 1,3 L-FucNAc α 1,3 D-FucNAc) n 1
β 1,4 (D-ManNAcA β 1,4 L-FucNAc 3 OAc α 1,3 D-FucNAc) n 1
α 1,3 (D-ManNAcA 4 OAc β 1,3 L-FucNAc α 1,3 D-FucNAc) n 1

FIG.4



pLAFR	O11 *wbjA*::Tn50<*dhfr-1*>*wzy*::*cat*				
pEXT22	-	wbjA	Cap 8H*	Cap 5I*	Cap 5I
lane	1	2	3	4	5

FIG.5A



pLAFR	O11 Δ*wbjA* Δ*wzy*O11*						
pEXT22	Cap5IJH		Cap5IJ				
pACT3	-		Cap5H		-		
IPTG conc. mM	1	0.1	1	0.1	1	0.1	
Expression Temperature	37°C						30°C
lane	1	2	3	4	5	6	7

FIG.5B

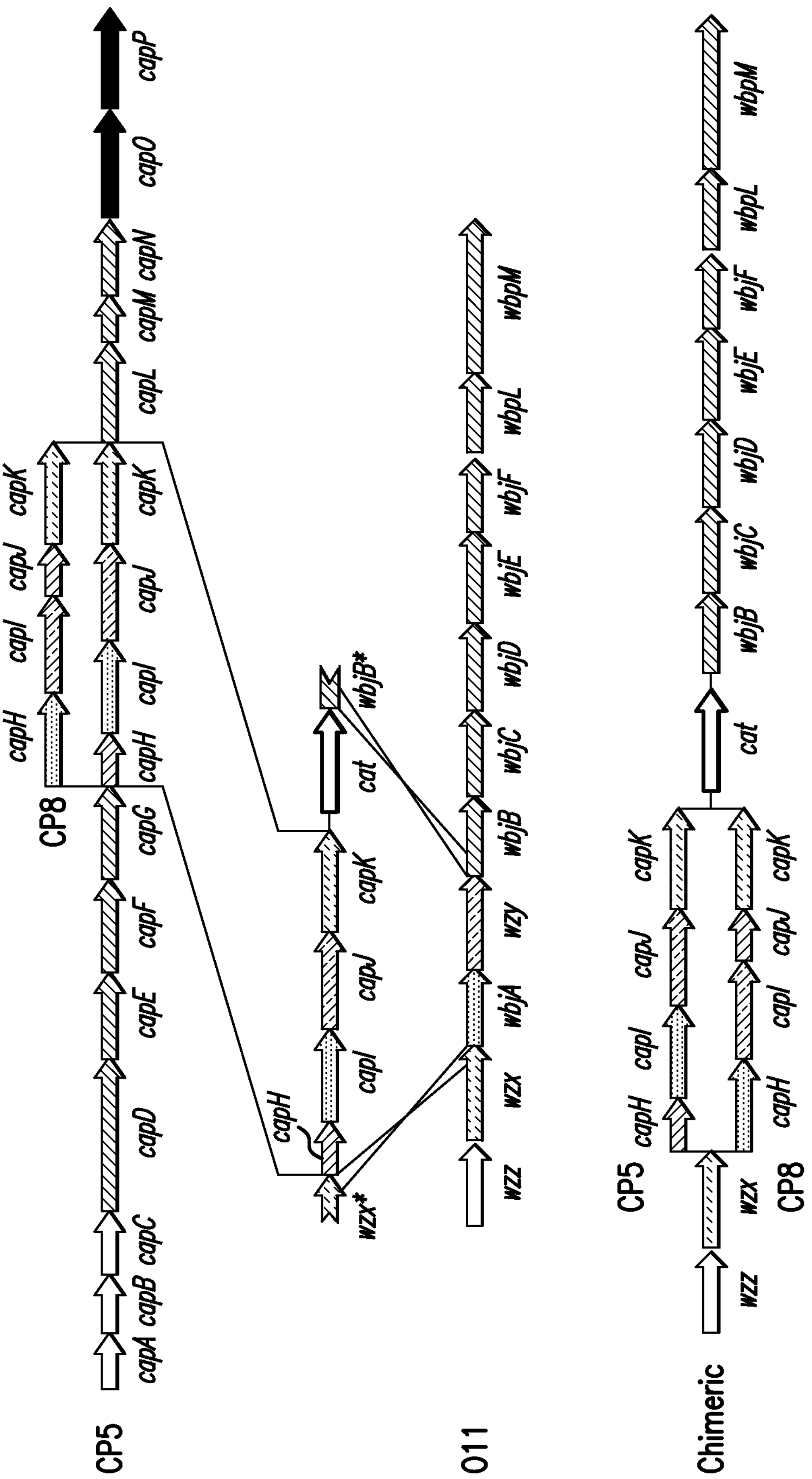

CP5
capA capB capC
capD
capE
capF
capG
capH
capI
capJ
capK
capL
capM capN
capO
capP
CP8
capH
capI
capJ
capK
capH
wzx*
capI
capJ
capK
cat
wbjB*
O11
wzz
wzx
wbjA
wzy
wbjB
wbjC
wbjD
wbjE
wbjF
wbpL
wbpM
Chimeric
wzz
CP5
wzx
CP8
capH
capI
capJ
capK
capH
capI
capJ
capK
cat
wbjB
wbjC
wbjD
wbjE
wbjF
wbpL
wbpM

FIG.6

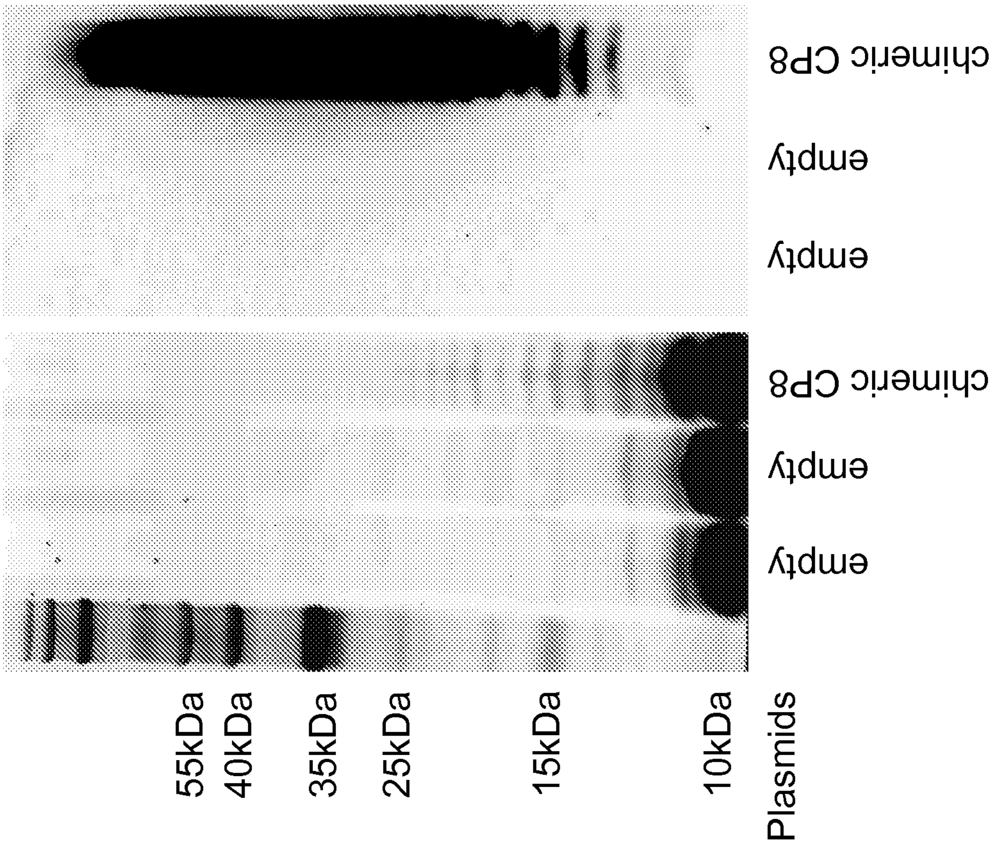

55kDa
40kDa
35kDa
25kDa
15kDa
10kDa
Plasmids
empty
empty
chimeric CP8
empty
empty
chimeric CP8

FIG.7B

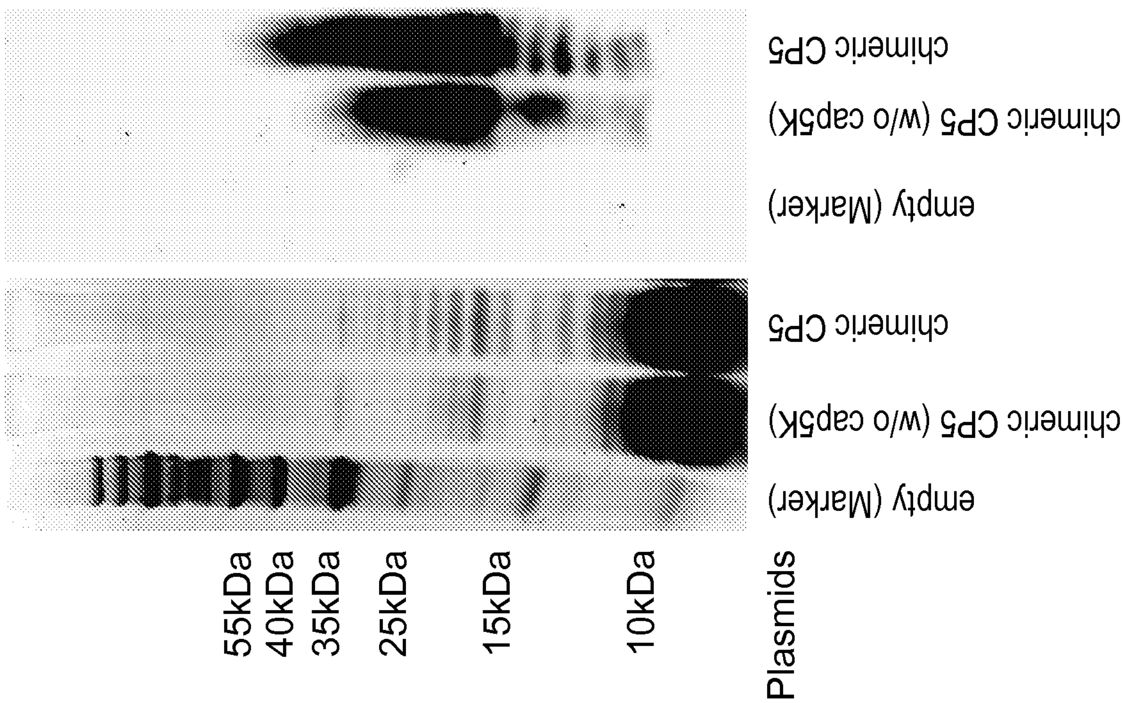

55kDa
40kDa
35kDa
25kDa
15kDa
10kDa
Plasmids
empty (Marker)
chimeric CP5 (w/o cap5K)
chimeric CP5
empty (Marker)
chimeric CP5 (w/o cap5K)
chimeric CP5

FIG.7A

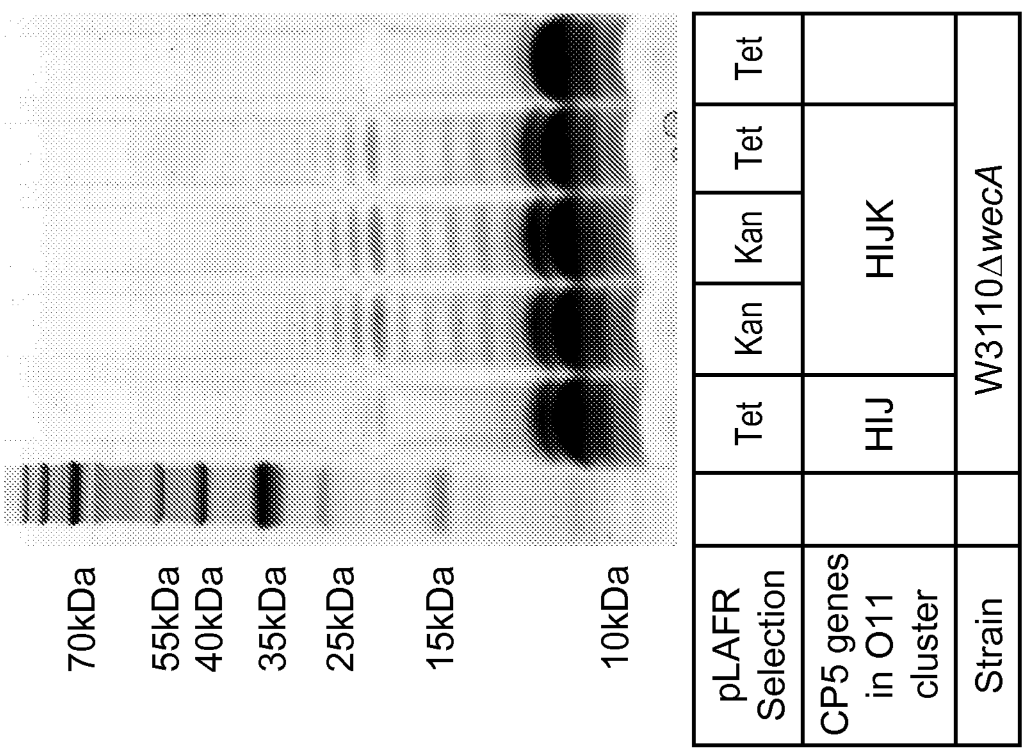

70kDa
55kDa
40kDa
35kDa
25kDa
15kDa
10kDa
pLAFR Selection
Tet
Kan
Kan
Tet
Tet
CP5 genes in O11 cluster
HIJ
HIJK
Strain
W3110ΔwecA

FIG.8A

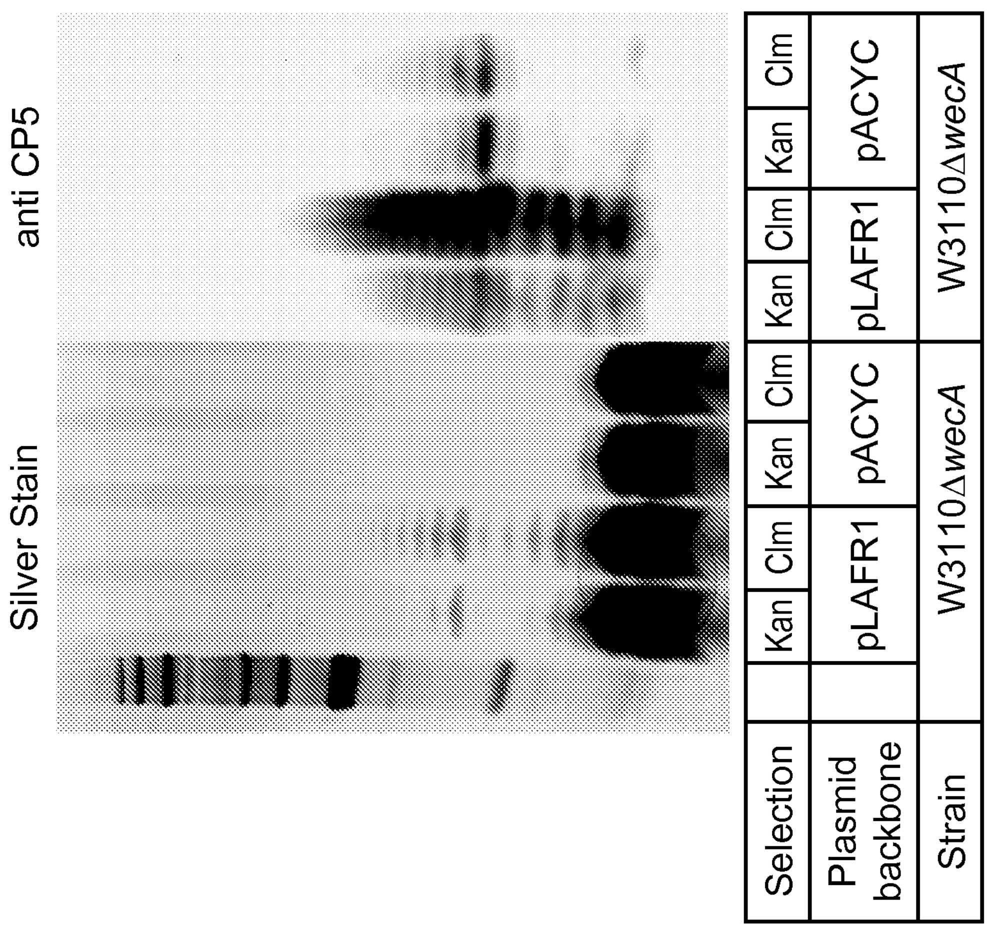
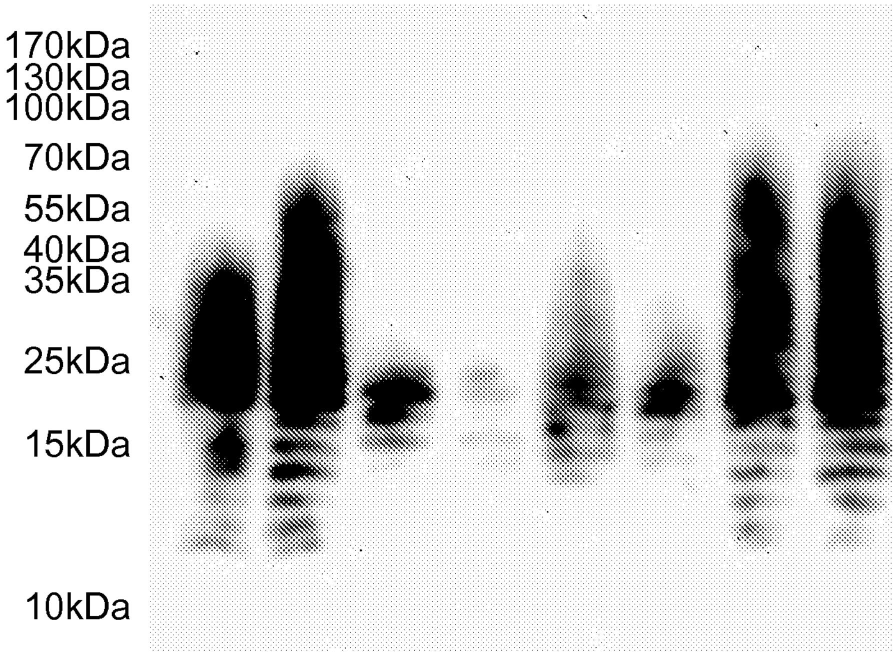

Silver Stain
anti CP5
Selection
Kan
Clm
Kan
Clm
Kan
Clm
Kan
Clm
Plasmid backbone
pLAFR1
pACYC
pLAFR1
pACYC
Strain
W3110ΔwecA
W3110ΔwecA

FIG.8B



CP5 Genes in O11 cluster	HIJ	HIJK	HIJ	KHIJ	HIJK	HIJ	KHIJ	HIJK
Promoter	*wzz/wzx*		-			PO121		
Strain	W3110 Δ*wecA*							

FIG.9

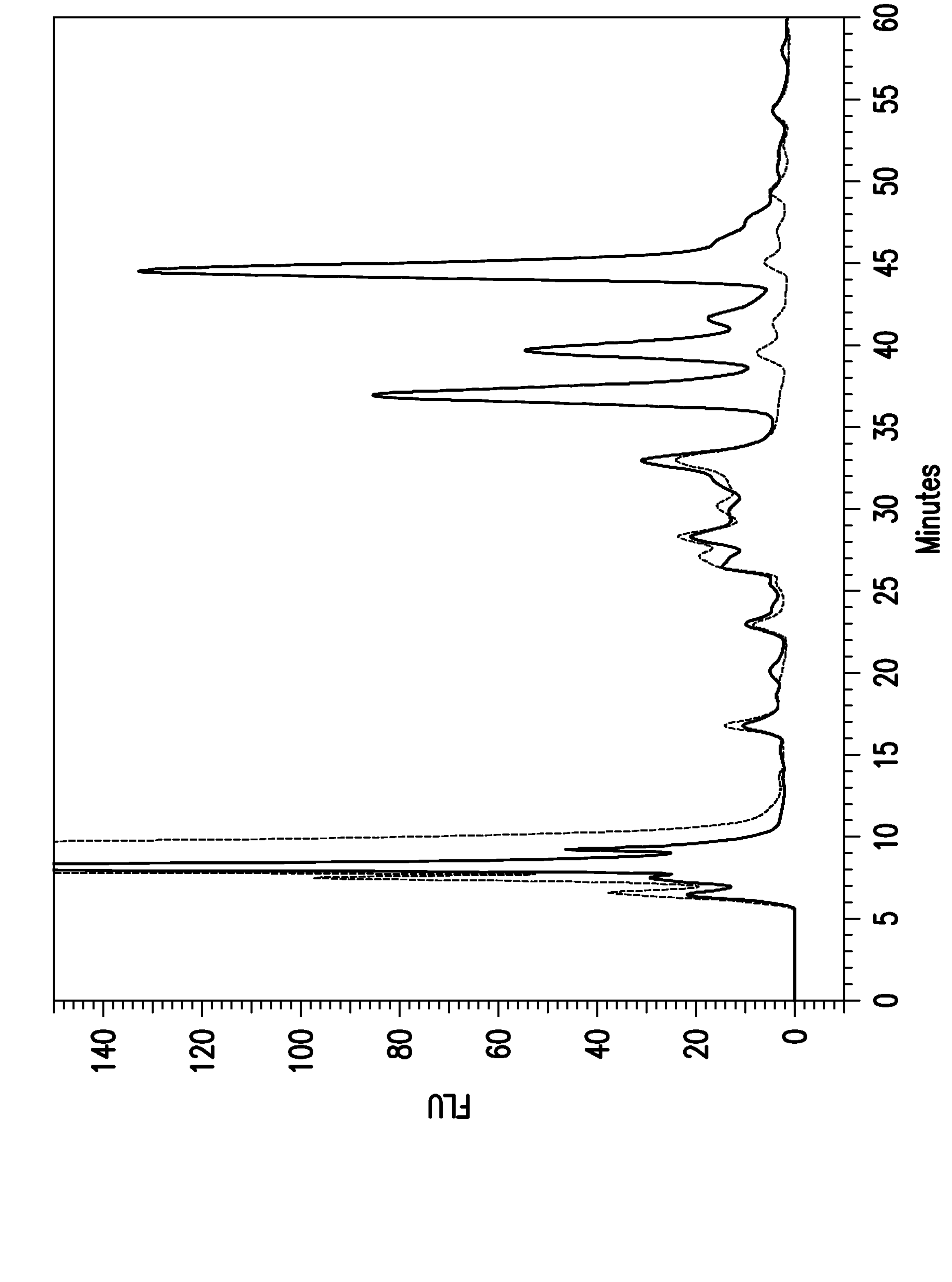

FLU
140
120
100
80
60
40
20
0
Minutes
0
5
10
15
20
25
30
35
40
45
50
55
60

FIG.10A

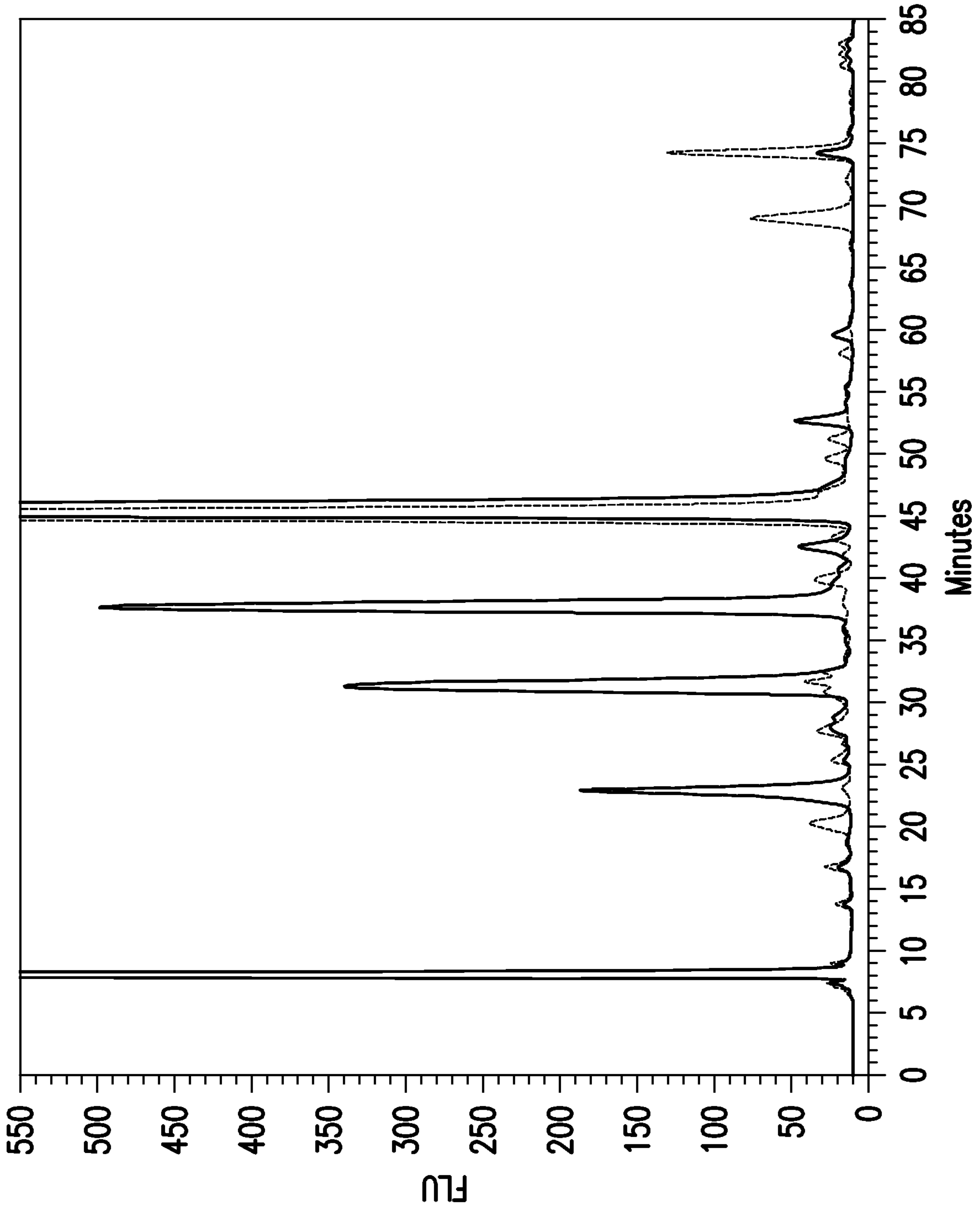

FLU
550
500
450
400
350
300
250
200
150
100
50
0
Minutes
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85

FIG.10B

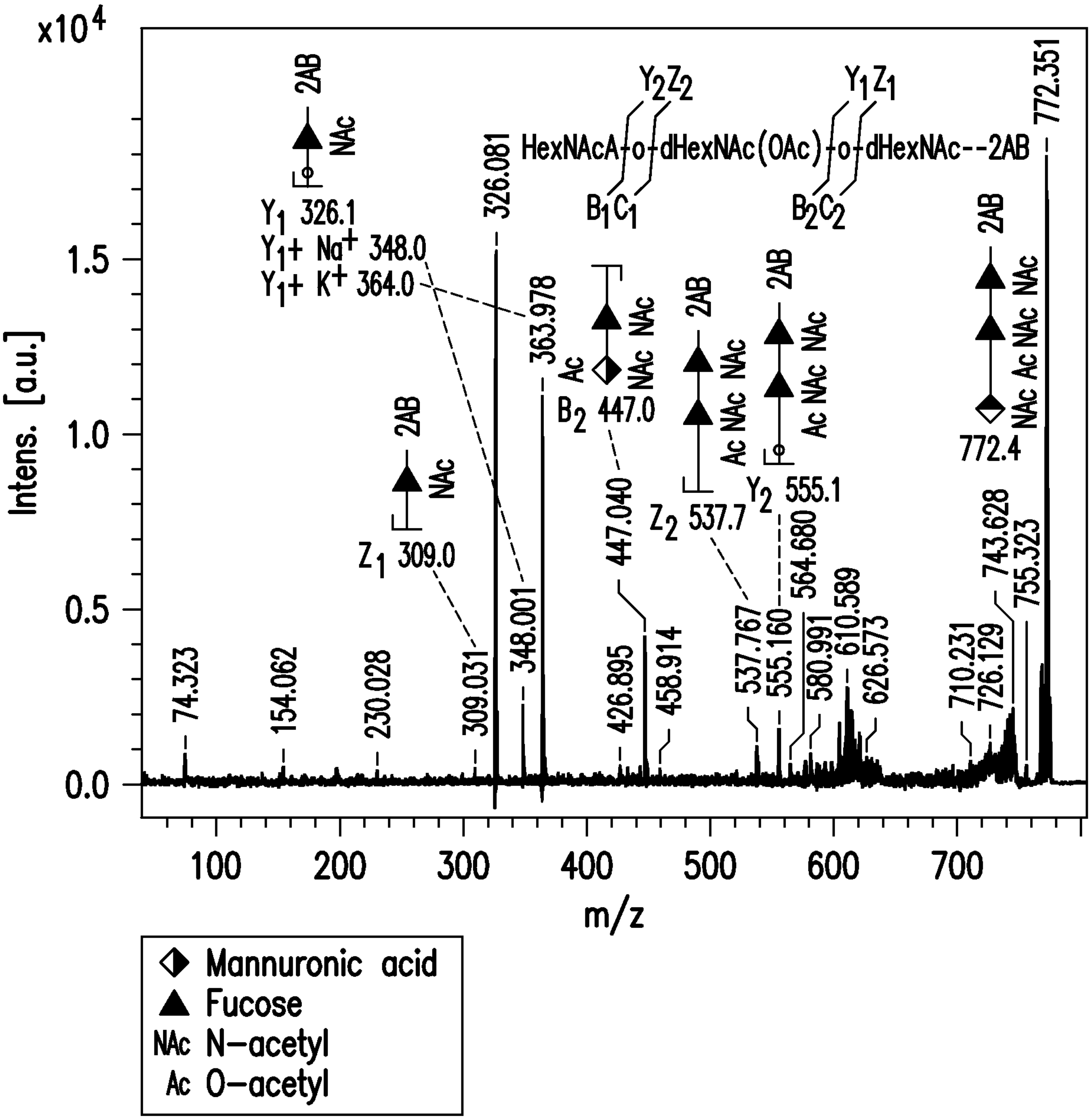

x10^4
Intens. [a.u.]
m/z
HexNAcA-o-dHexNAc(OAc)-o-dHexNAc--2AB
Y2Z2
Y1Z1
B1C1
B2C2
Y1 326.1
Y1+ Na+ 348.0
Y1+ K+ 364.0
Z1 309.0
B2 447.0
Z2 537.7
Y2 555.1
772.4
2AB
NAc
Ac
772.351
326.081
363.978
447.040
348.001
309.031
458.914
426.895
537.767
555.160
564.680
580.991
610.589
626.573
710.231
726.129
743.628
755.323
74.323
154.062
230.028
Mannuronic acid
Fucose
NAc N-acetyl
Ac O-acetyl

FIG.11A

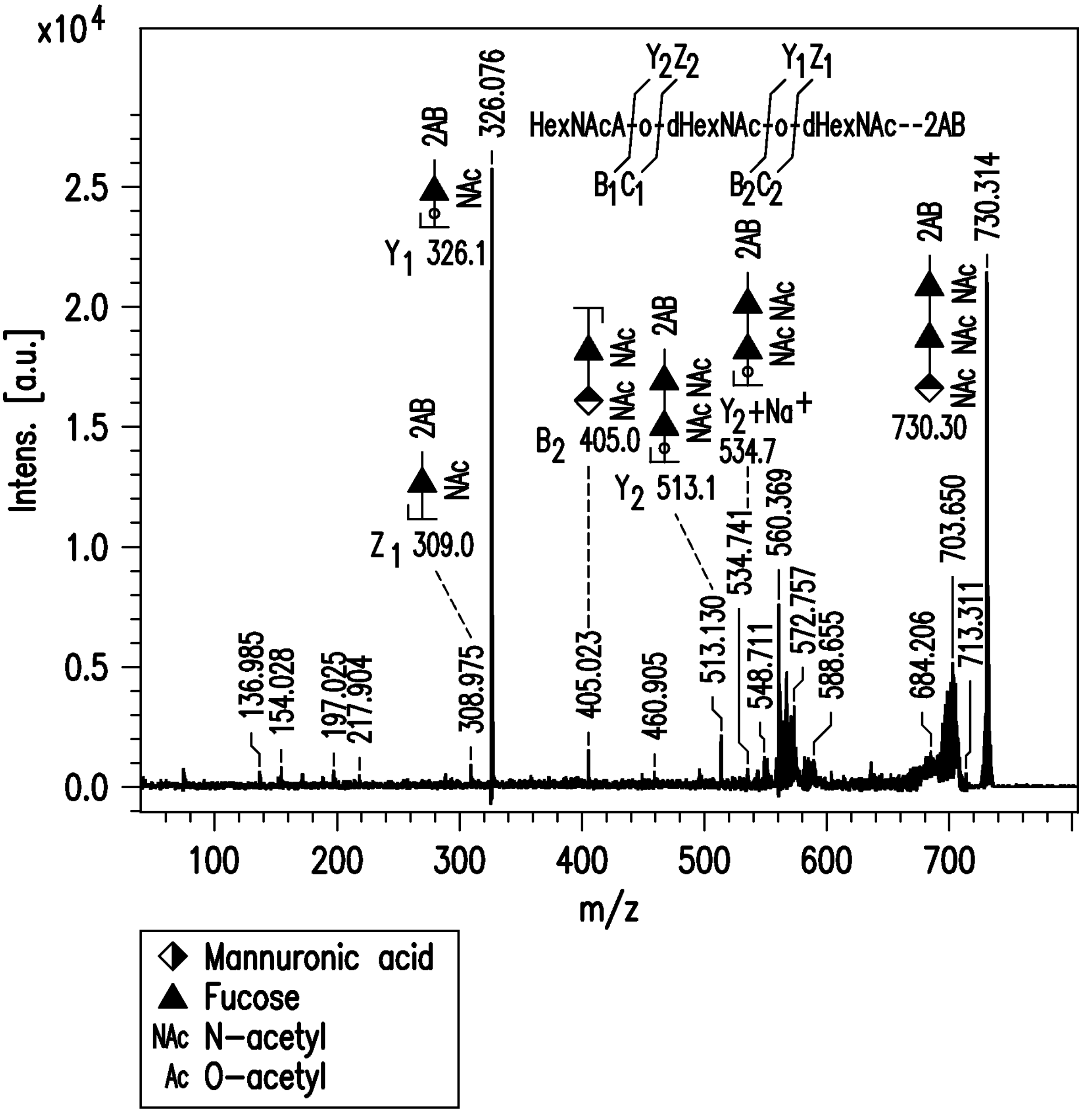

x10^4
Intens. [a.u.]
m/z
HexNAcA-o-dHexNAc-o-dHexNAc--2AB
Y2Z2
Y1Z1
B1C1
B2C2
326.076
Y1 326.1
Z1 309.0
B2 405.0
Y2 513.1
Y2+Na+
534.7
730.30
730.314
703.650
713.311
684.206
560.369
572.757
588.655
534.741
548.711
513.130
460.905
405.023
308.975
136.985
154.028
197.025
217.904
2AB
NAc
Mannuronic acid
Fucose
NAc N-acetyl
Ac O-acetyl

FIG.11B

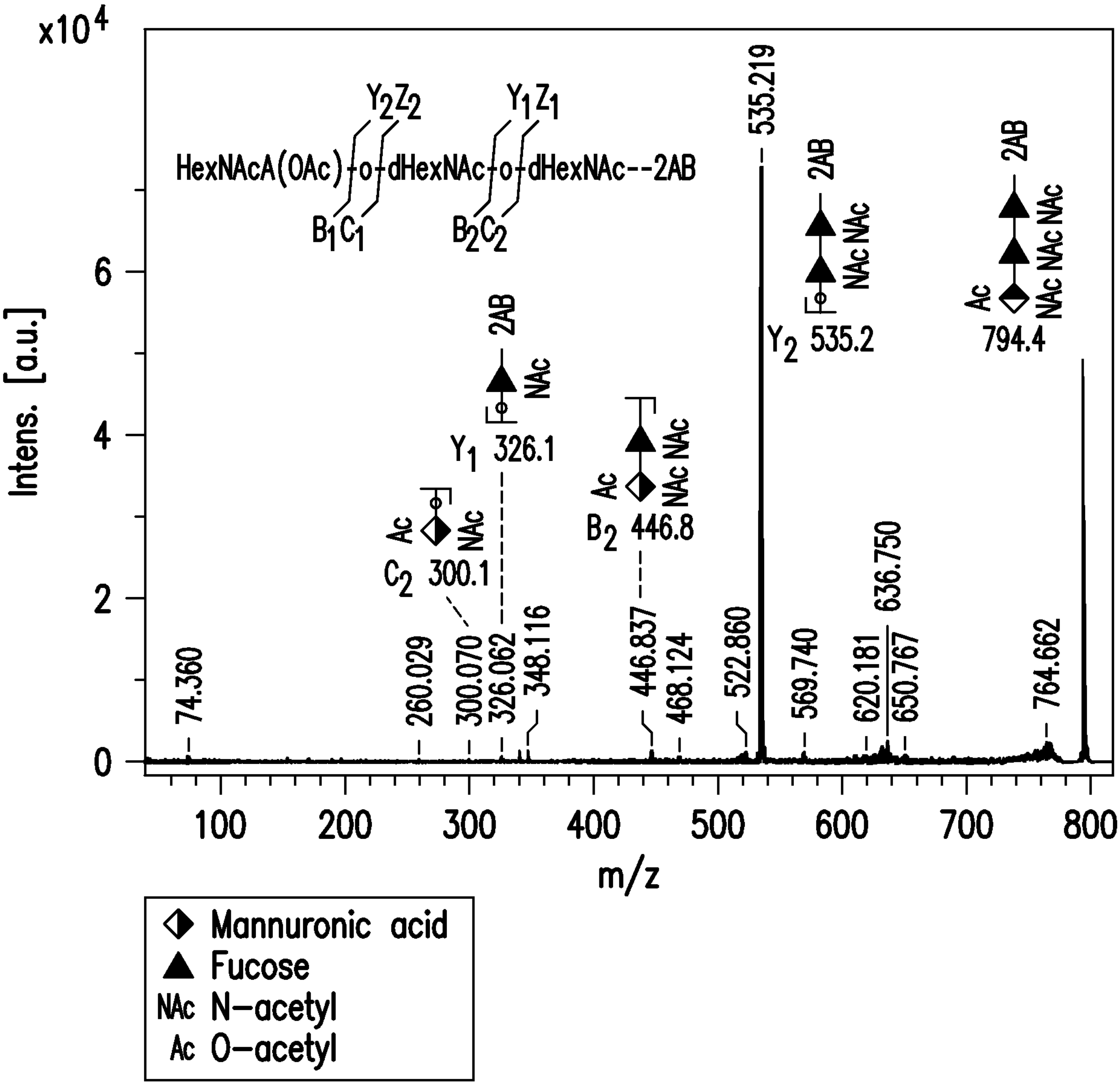

x10^4
Intens. [a.u.]
m/z
HexNAcA(OAc)-o-dHexNAc-o-dHexNAc--2AB
Y2Z2
Y1Z1
B1C1
B2C2
535.219
Y2 535.2
794.4
Y1 326.1
B2 446.8
C2 300.1
2AB
NAc
Ac
74.360
260.029
300.070
326.062
348.116
446.837
468.124
522.860
569.740
620.181
636.750
650.767
764.662
Mannuronic acid
Fucose
NAc N-acetyl
Ac O-acetyl

FIG.11C

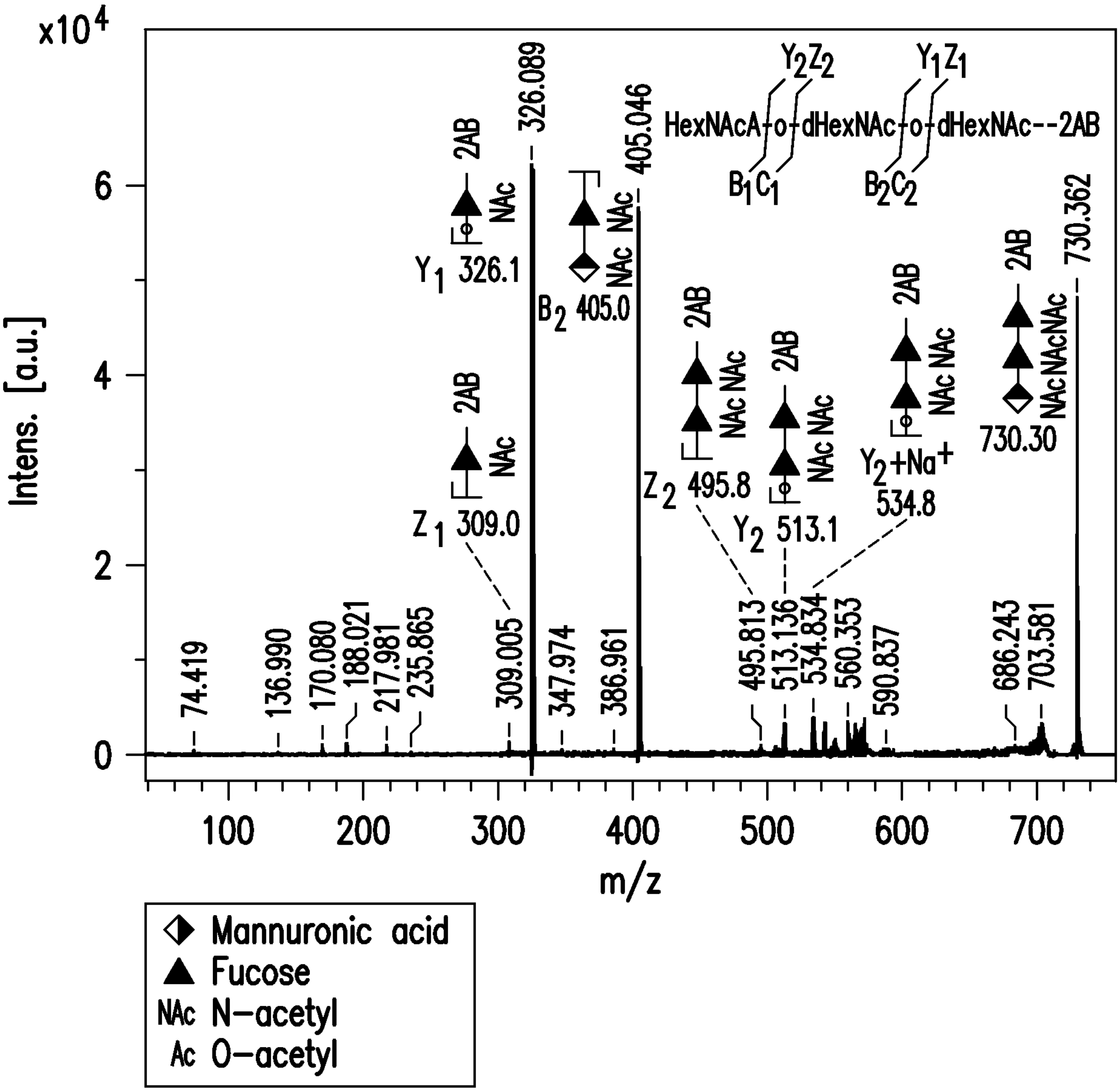

x10^4
Intens. [a.u.]
m/z
HexNAcA-o-dHexNAc-o-dHexNAc--2AB
Y2Z2
Y1Z1
B1C1
B2C2
Y1 326.1
B2 405.0
Z1 309.0
Z2 495.8
Y2 513.1
Y2+Na+ 534.8
730.30
326.089
405.046
730.362
74.419
136.990
170.080
188.021
217.981
235.865
309.005
347.974
386.961
495.813
513.136
534.834
560.353
590.837
686.243
703.581
Mannuronic acid
Fucose
NAc N-acetyl
Ac O-acetyl

FIG.11D

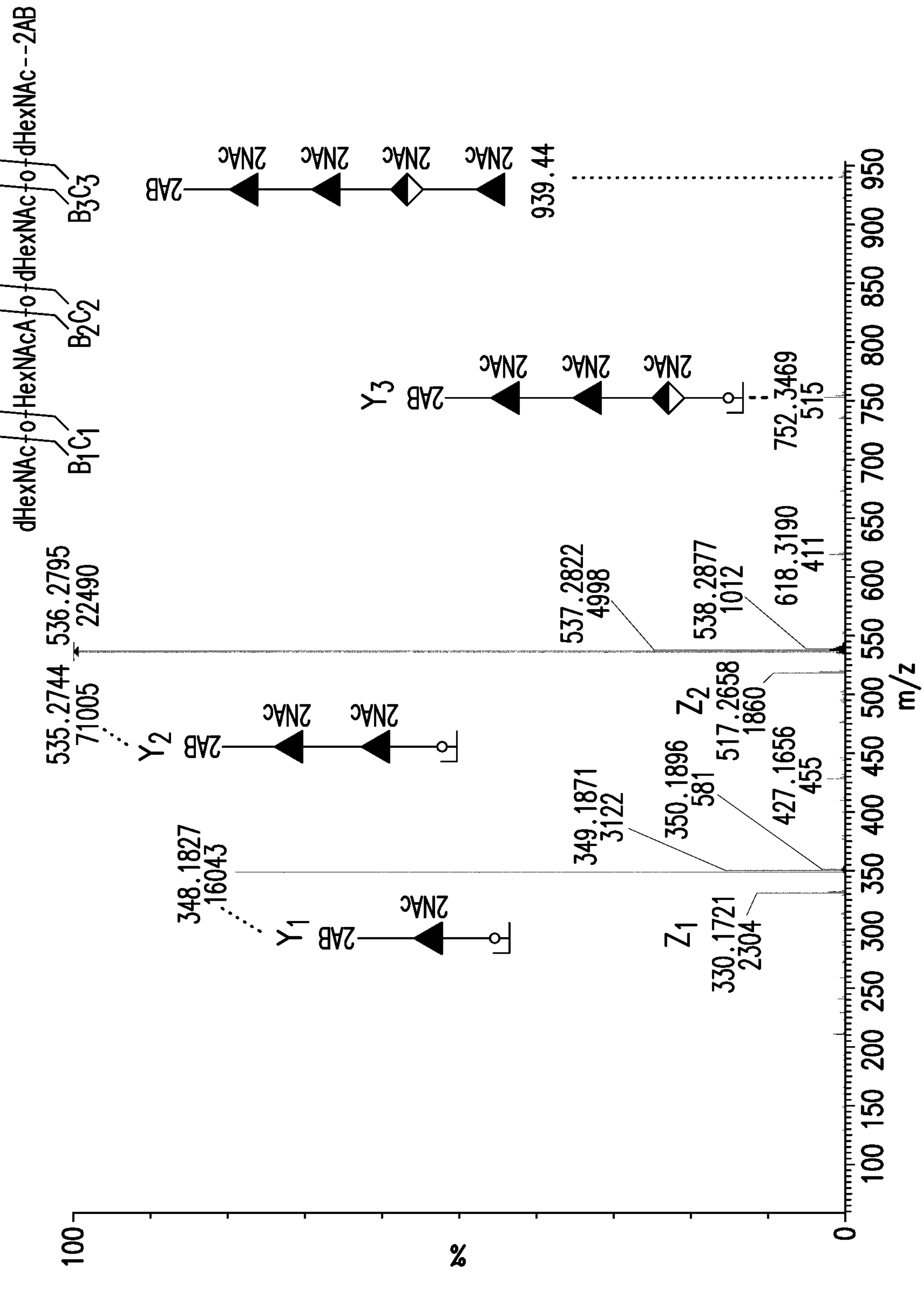

dHexNAc-o-HexNAc-o-HexNAcA-o-dHexNAc-o-dHexNAc--2AB
Y3Z3
B1C1
Y2Z2
B2C2
Y1Z1
B3C3
2AB
2NAc
939.44
Y3
752.3469
515
618.3190
411
536.2795
22490
537.2822
4998
538.2877
1012
535.2744
71005
Y2
Z2
517.2658
1860
350.1896
581
427.1656
455
348.1827
16043
349.1871
3122
Y1
Z1
330.1721
2304
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950

FIG.11E

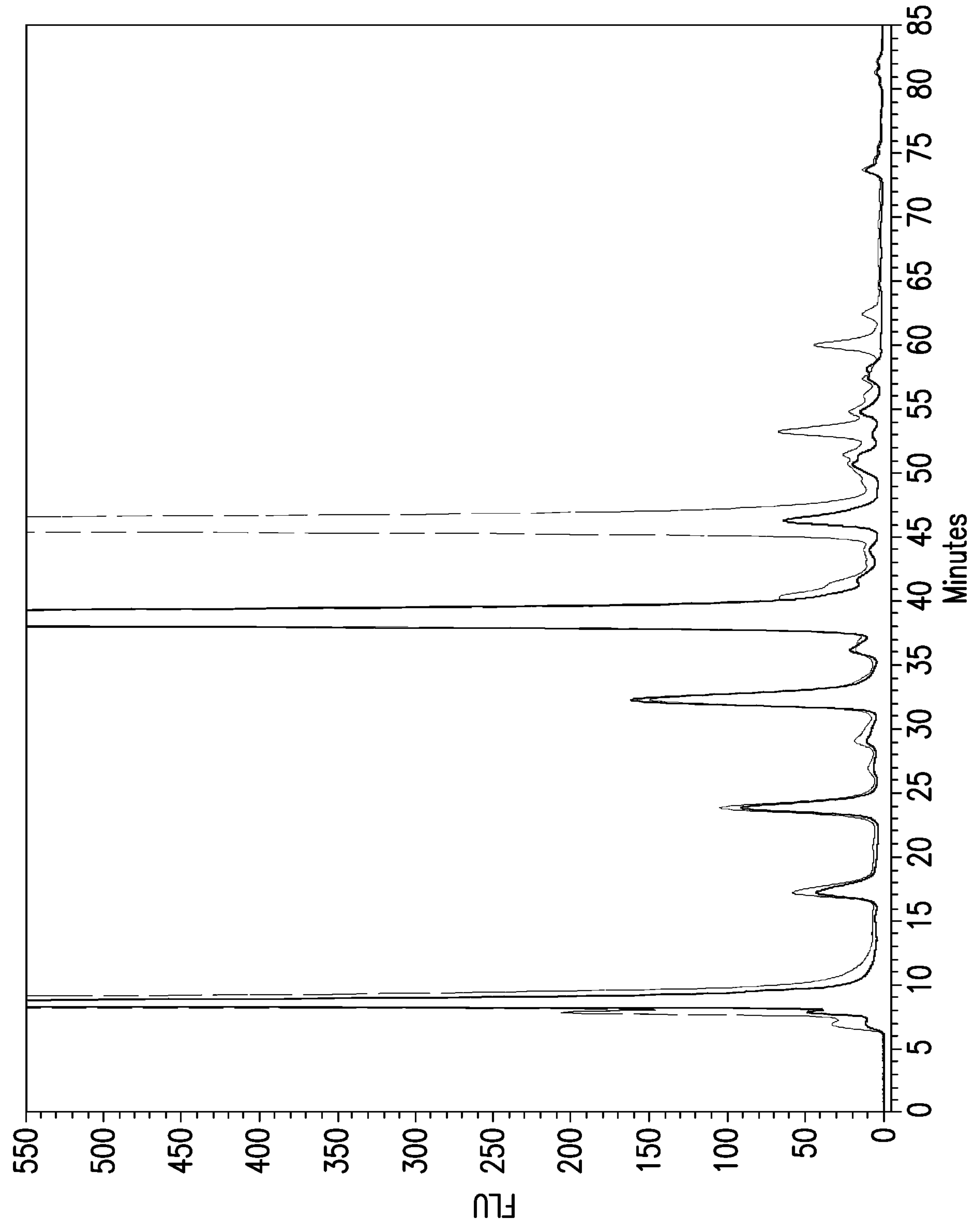


FIG.11F

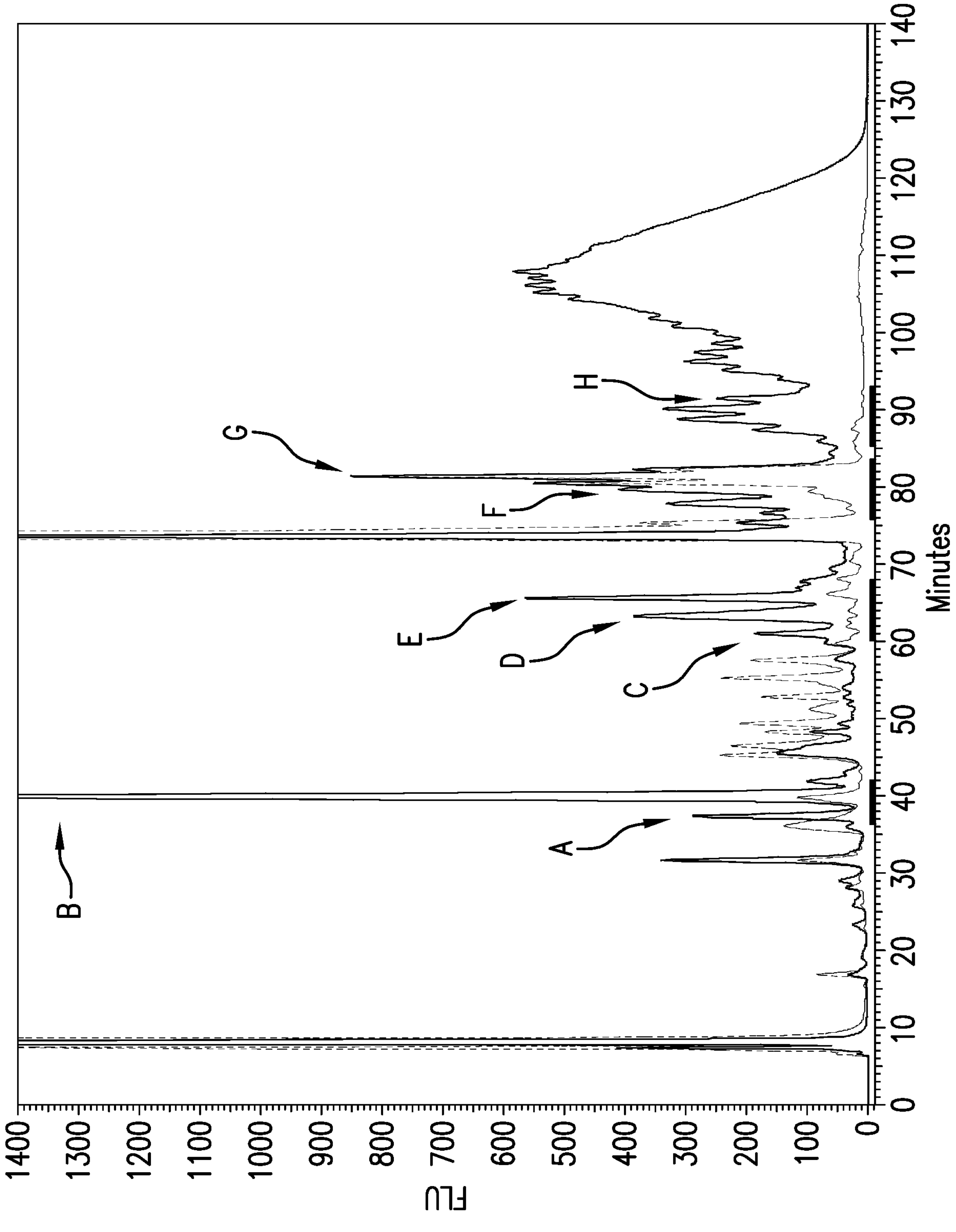

FLU
Minutes
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
10
20
30
40
50
60
70
80
90
100
110
120
130
140
A
B
C
D
E
F
G
H

FIG.11G

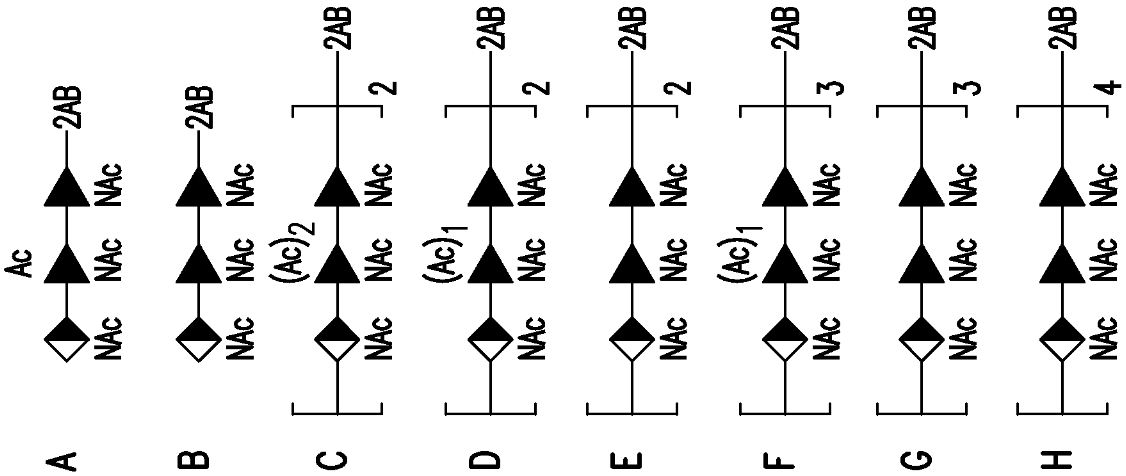


FIG.11G-1

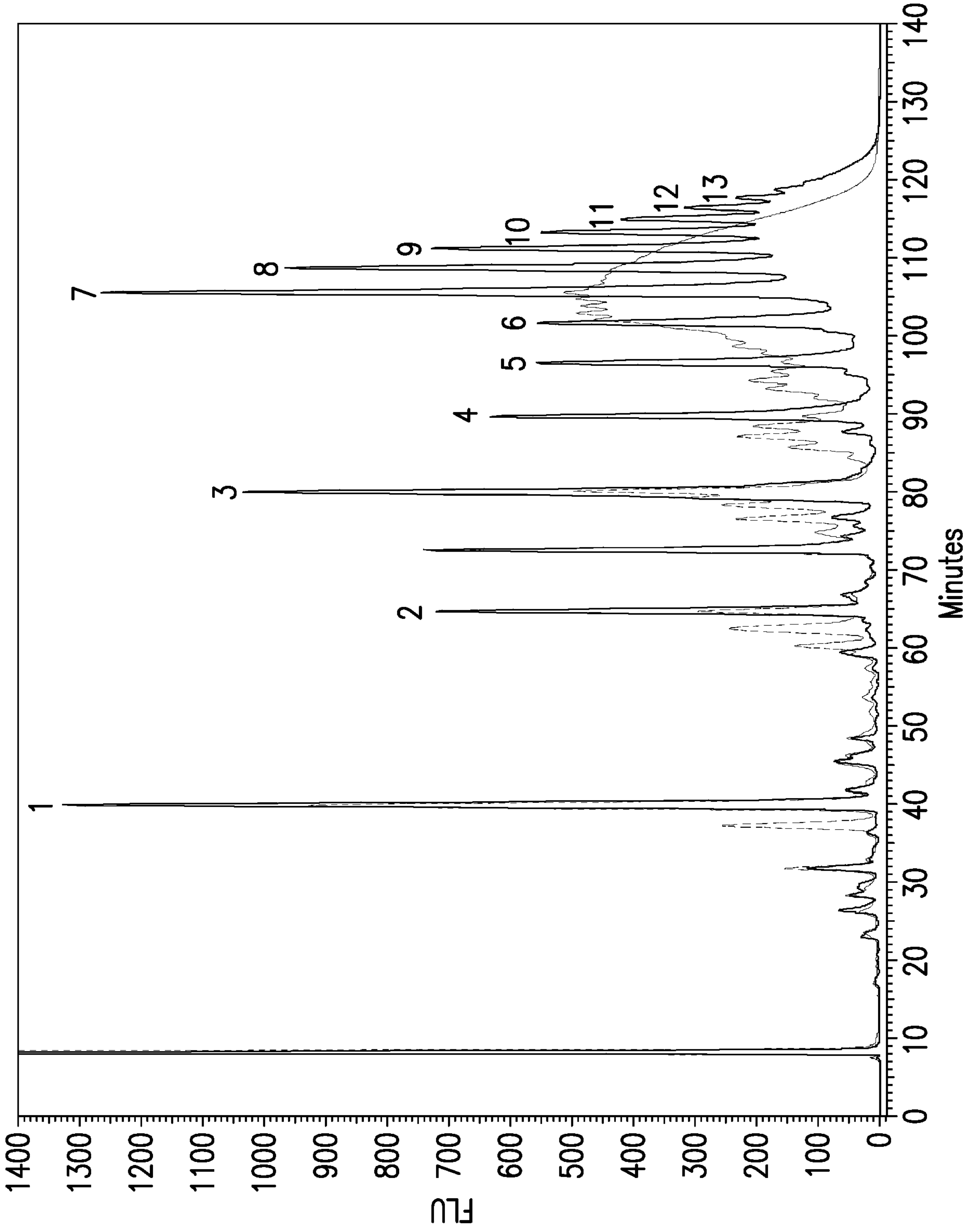

FLU
Minutes
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
10
20
30
40
50
60
70
80
90
100
110
120
130
140
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG.11H

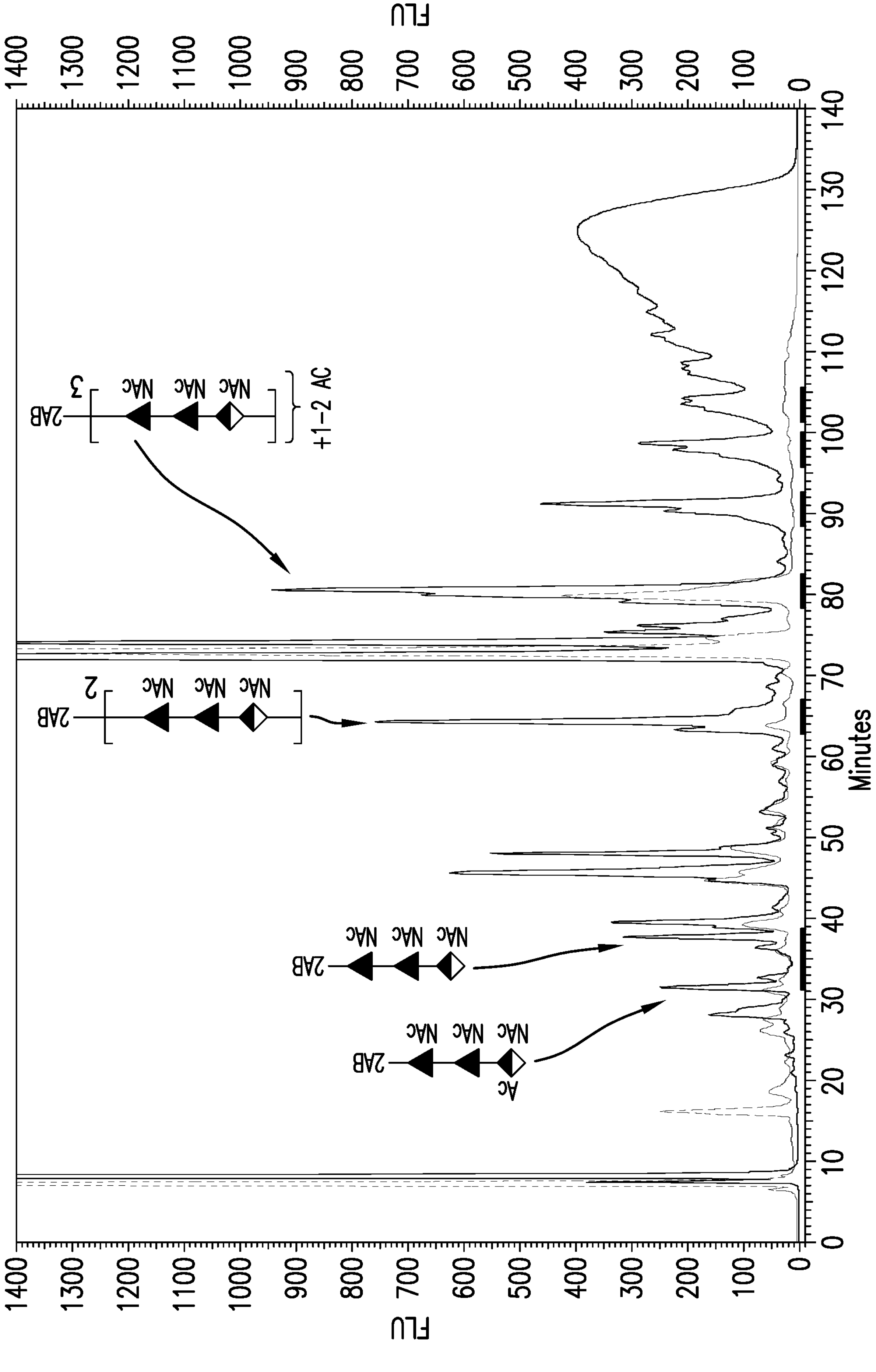


FIG.11I

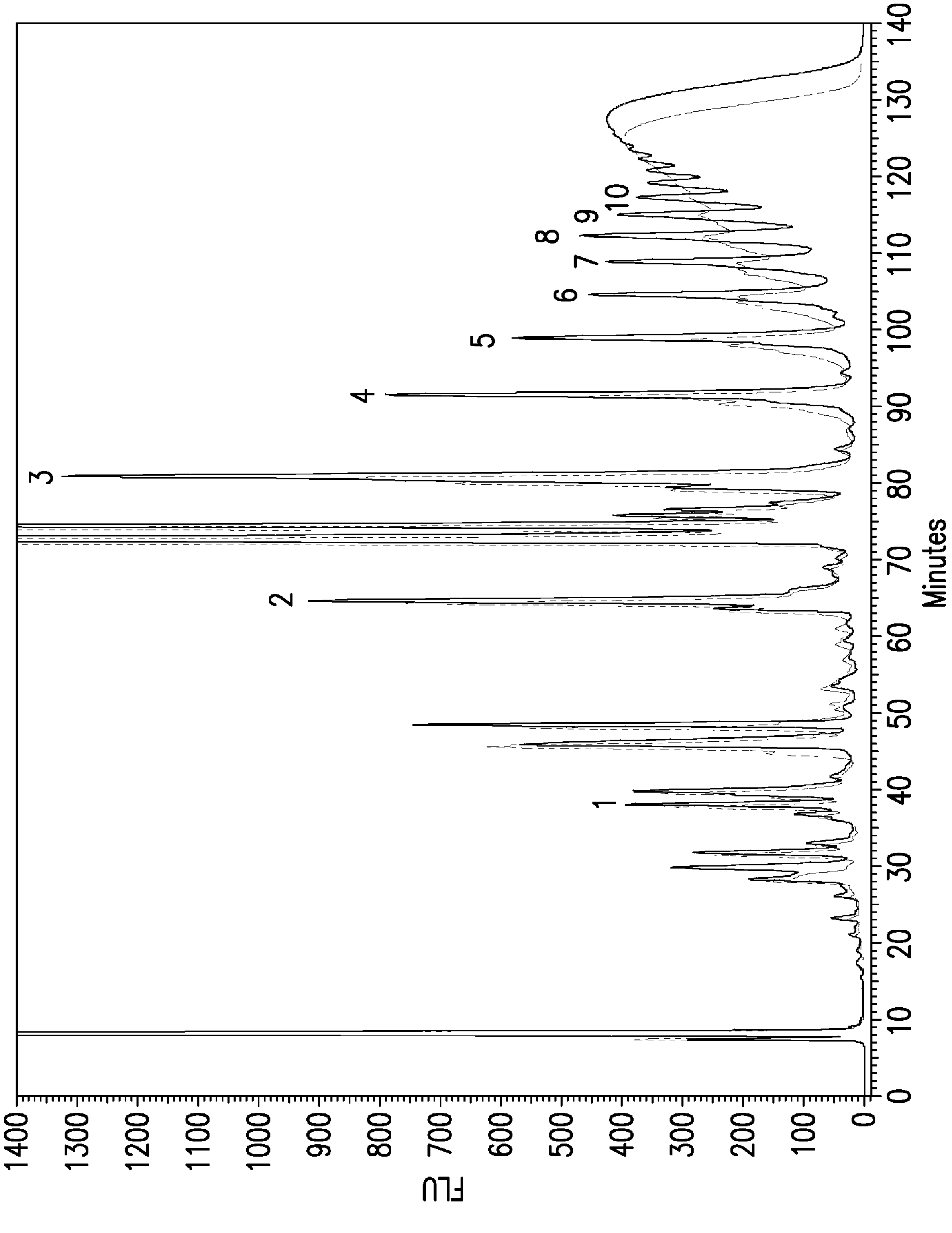

FLU
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Minutes
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
1
2
3
4
5
6
7
8
9
10

FIG.11J

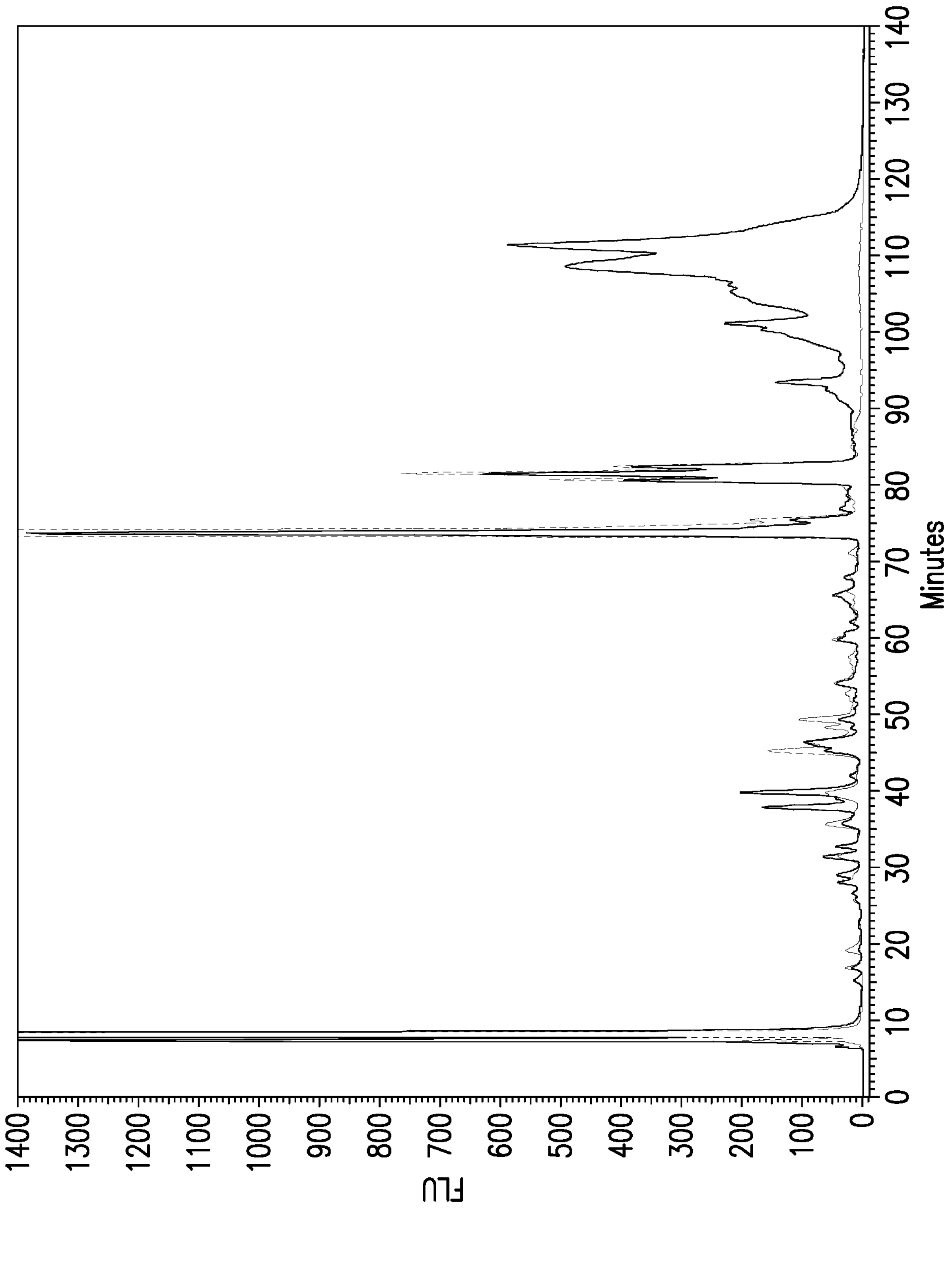

FLU
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
Minutes
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

FIG.11K

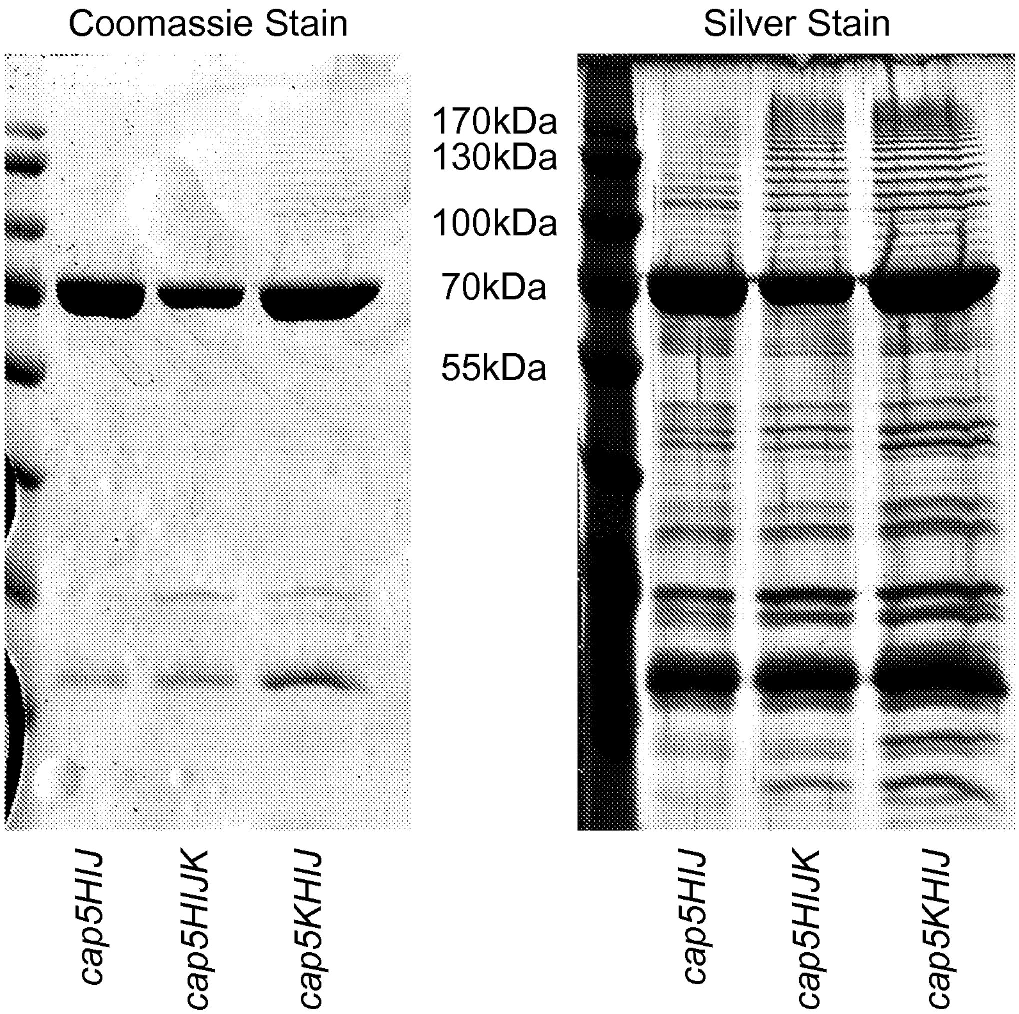

Coomassie Stain
Silver Stain
170kDa
130kDa
100kDa
70kDa
55kDa
cap5HIJ
cap5HIJK
cap5KHIJ
cap5HIJ
cap5HIJK
cap5KHIJ

FIG.12

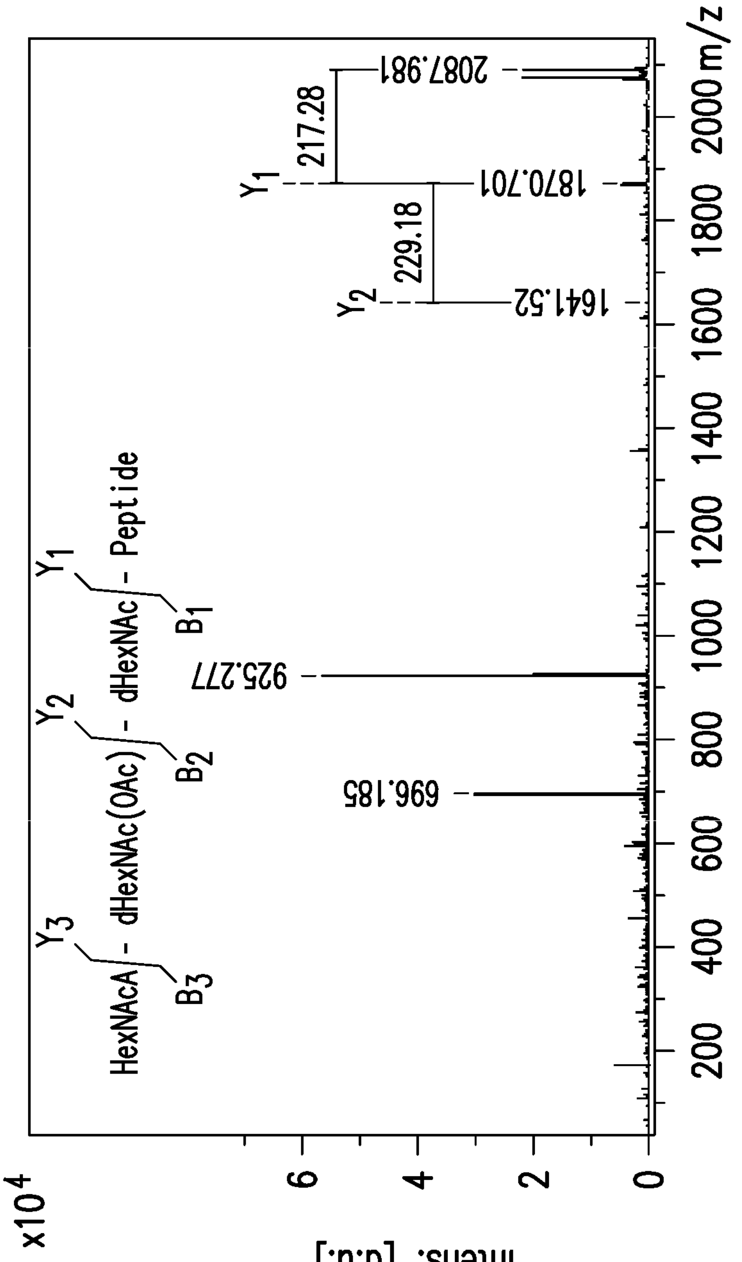


FIG.13B

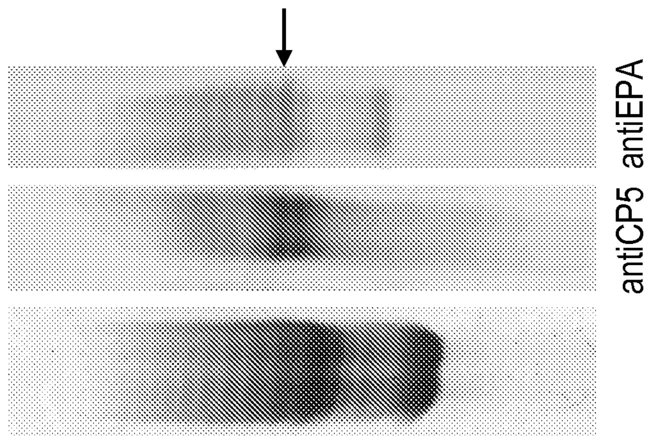


FIG.13A

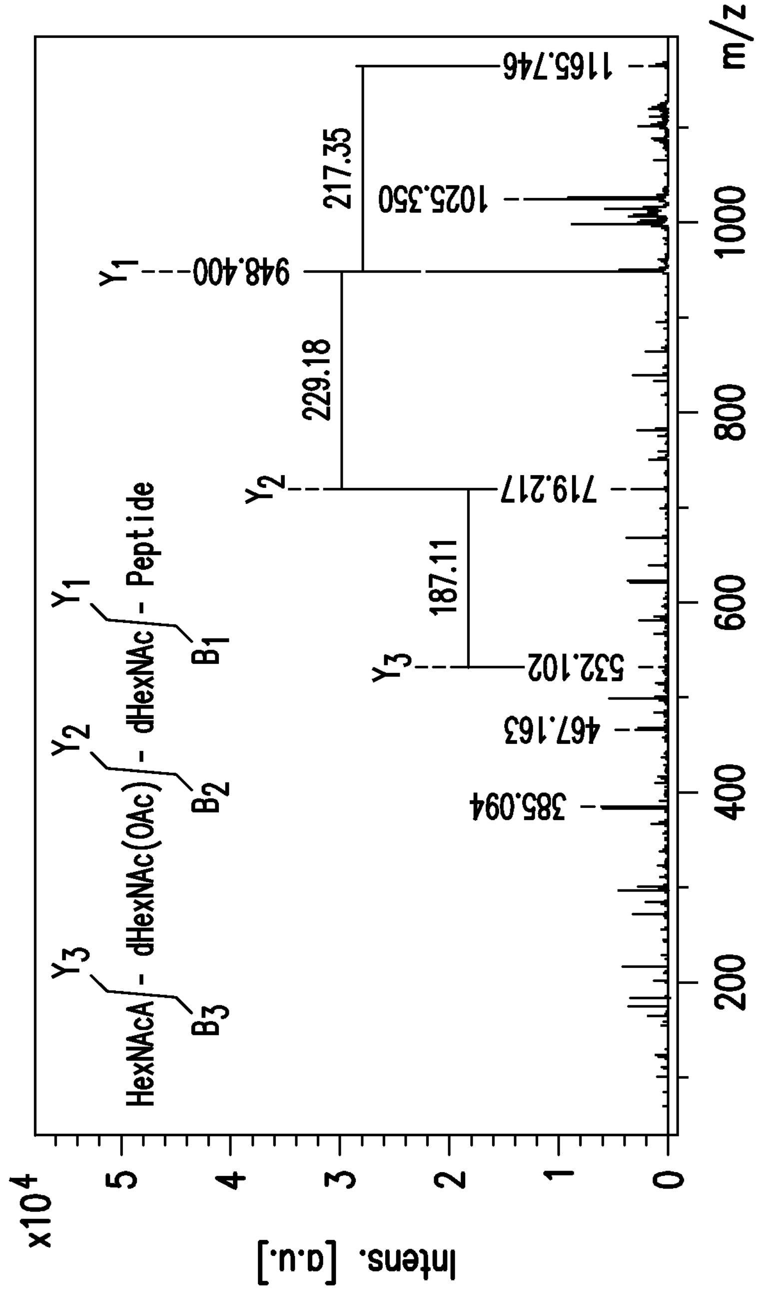

x10^4
Intens. [a.u.]
m/z
HexNAcA - dHexNAc(OAc) - dHexNAc - Peptide
Y3
Y2
Y1
B3
B2
B1
385.094
467.163
532.102
719.217
948.400
1025.350
1165.746
187.11
229.18
217.35
0
1
2
3
4
5
200
400
600
800
1000

FIG.13C

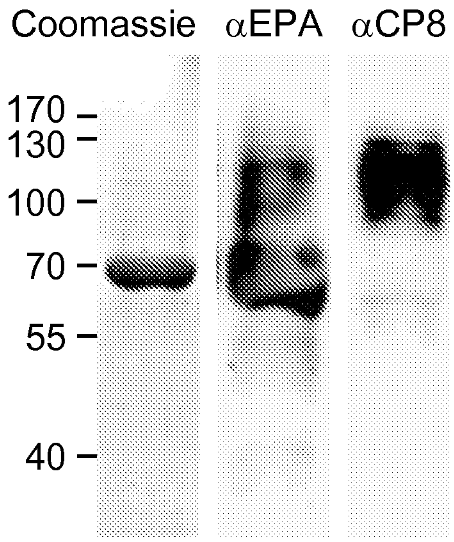
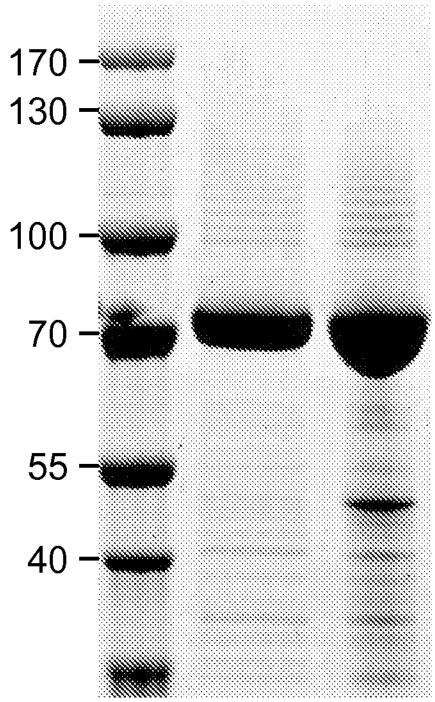


FIG.13D



Plasmid No.	3	2

FIG.13E

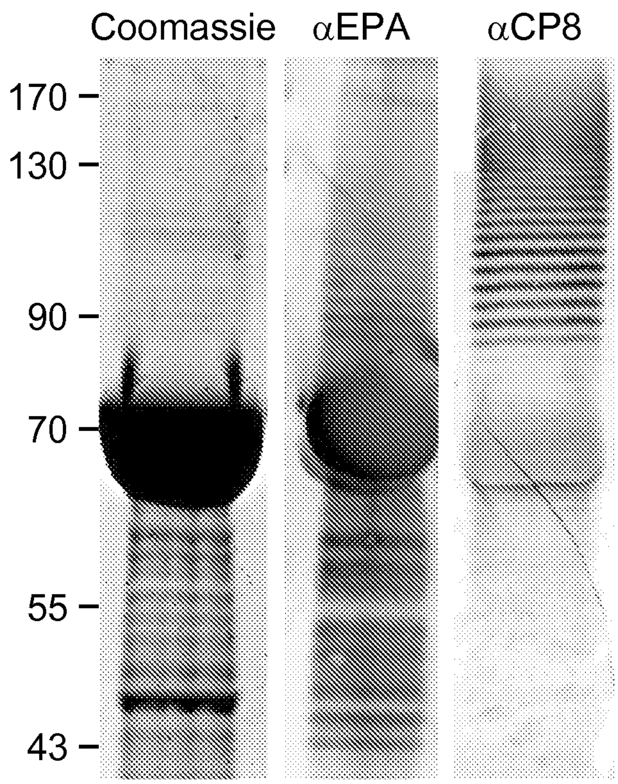


FIG.13F

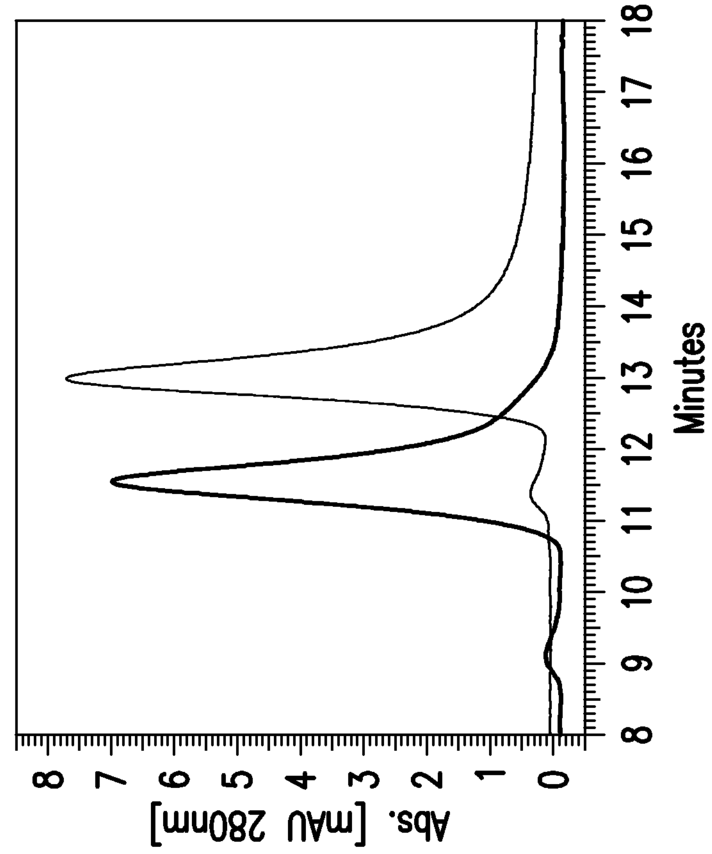


FIG.14B

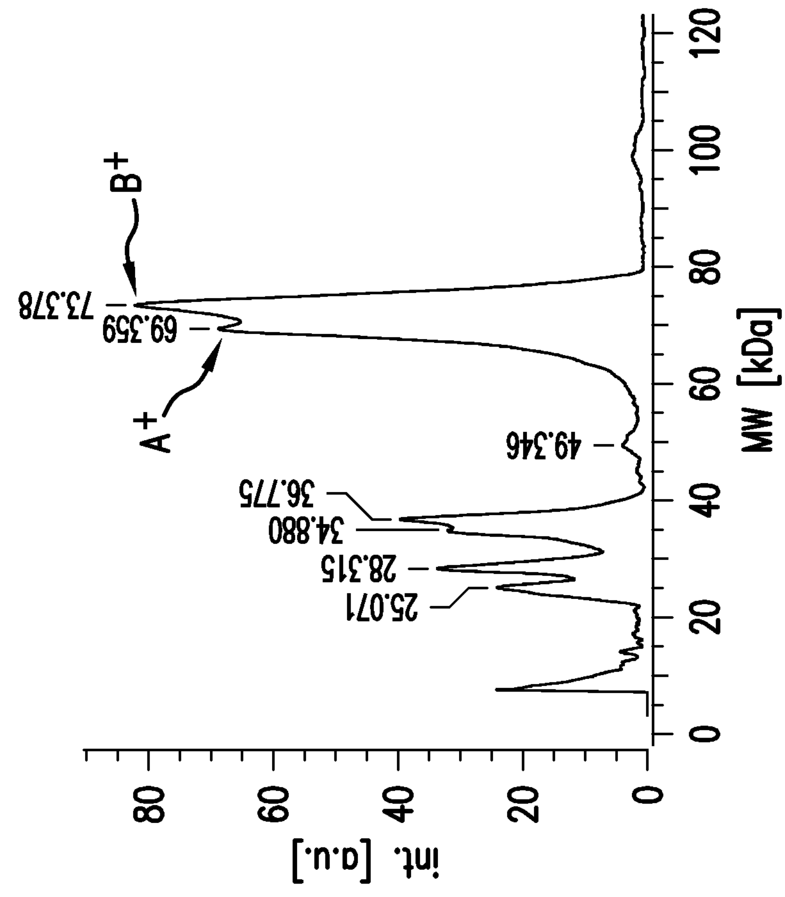


FIG.14A

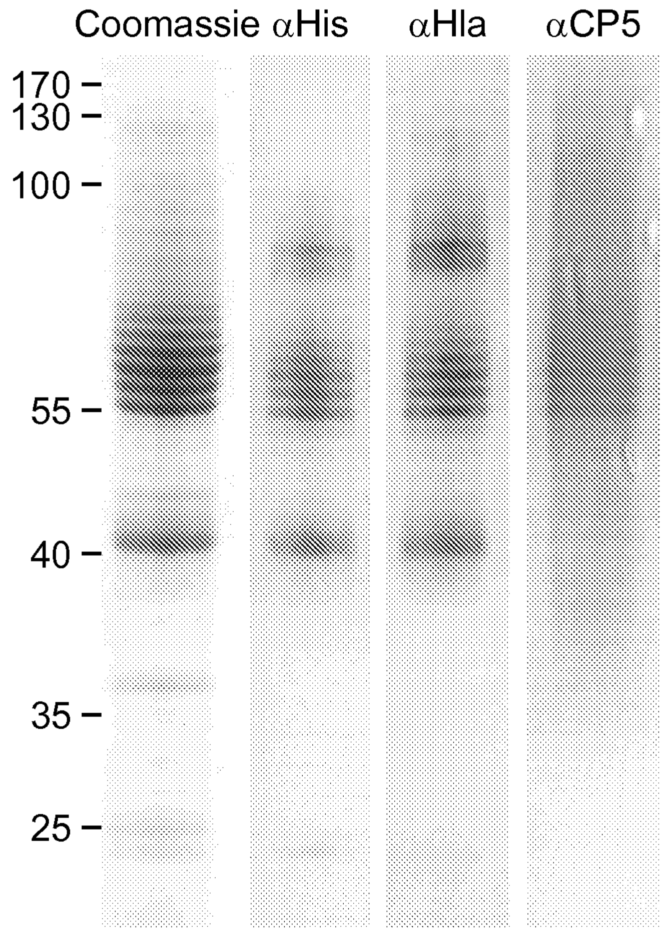


FIG.14C

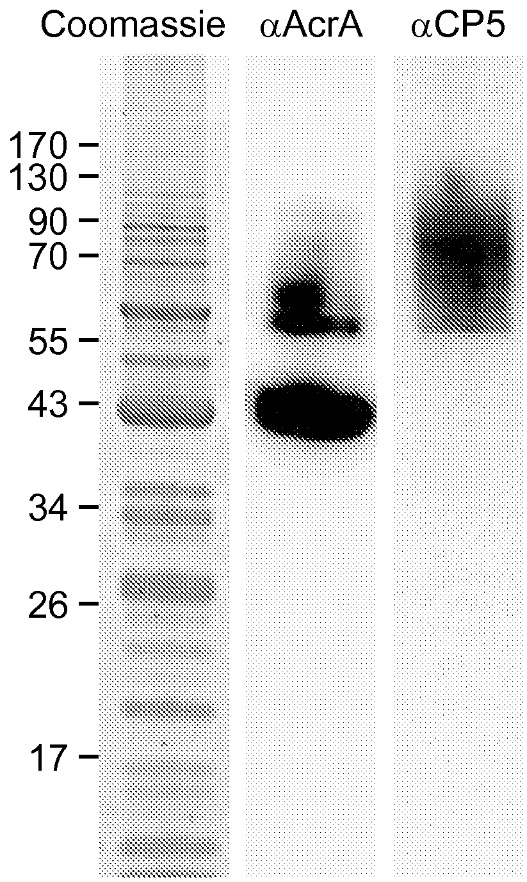


FIG.14D

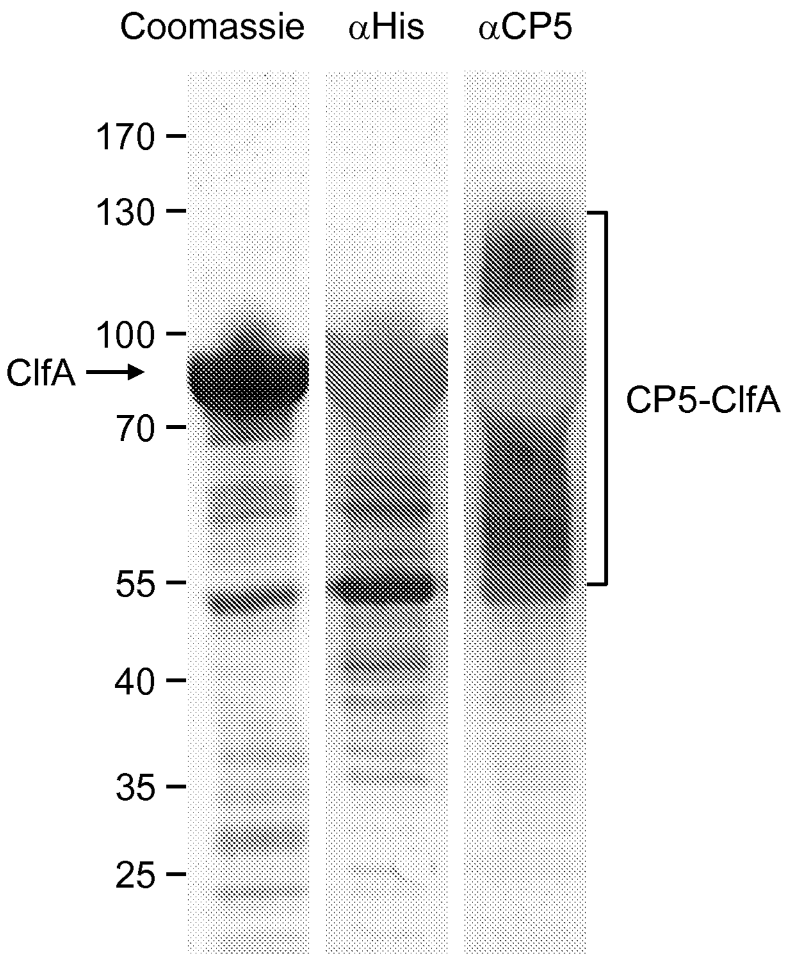


FIG.14E

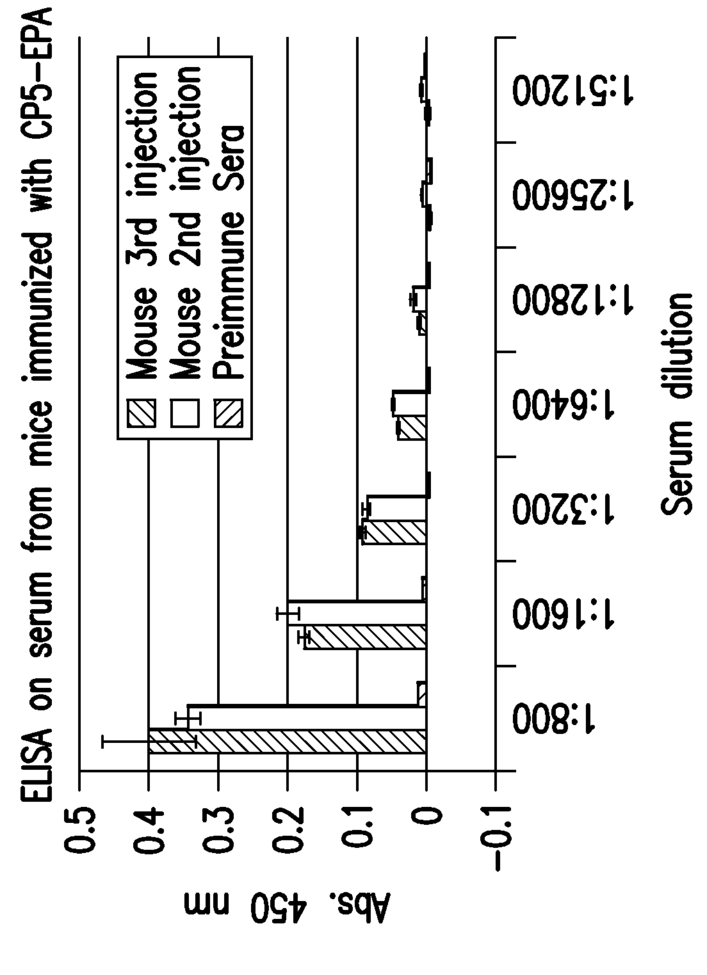


FIG.15A

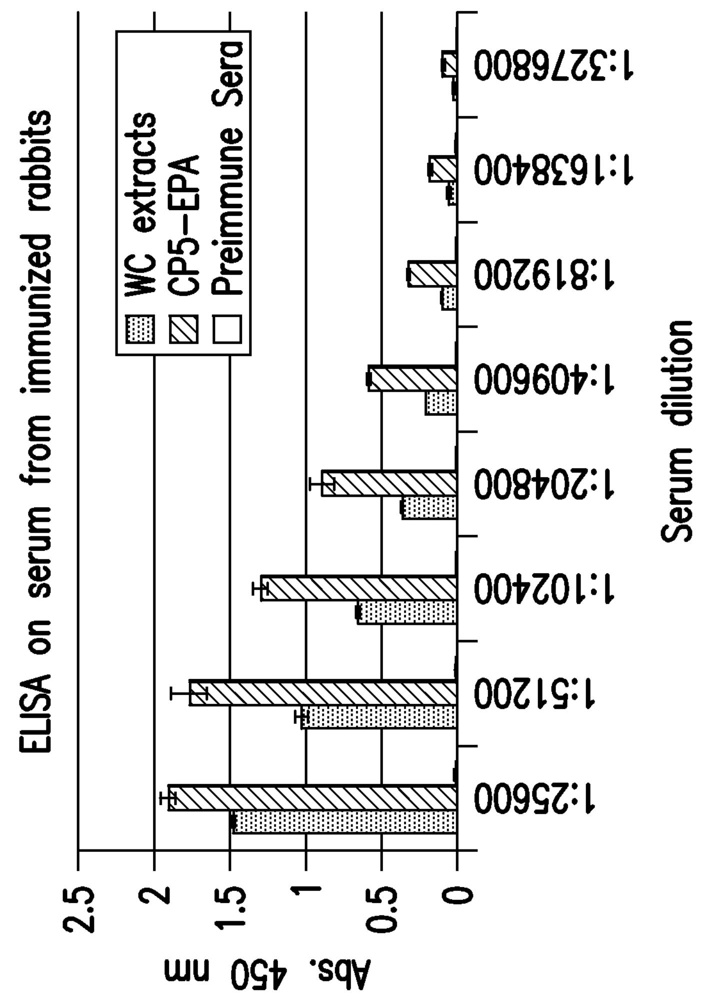


FIG.15B

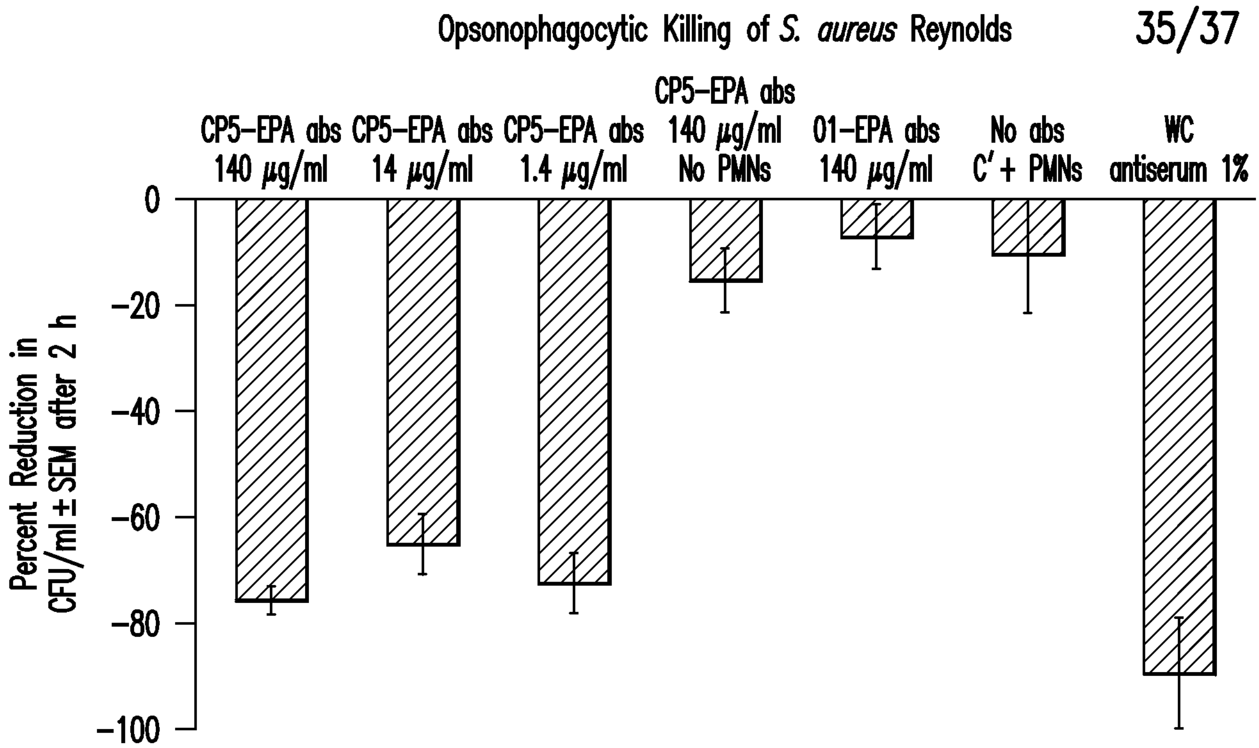


FIG.16A

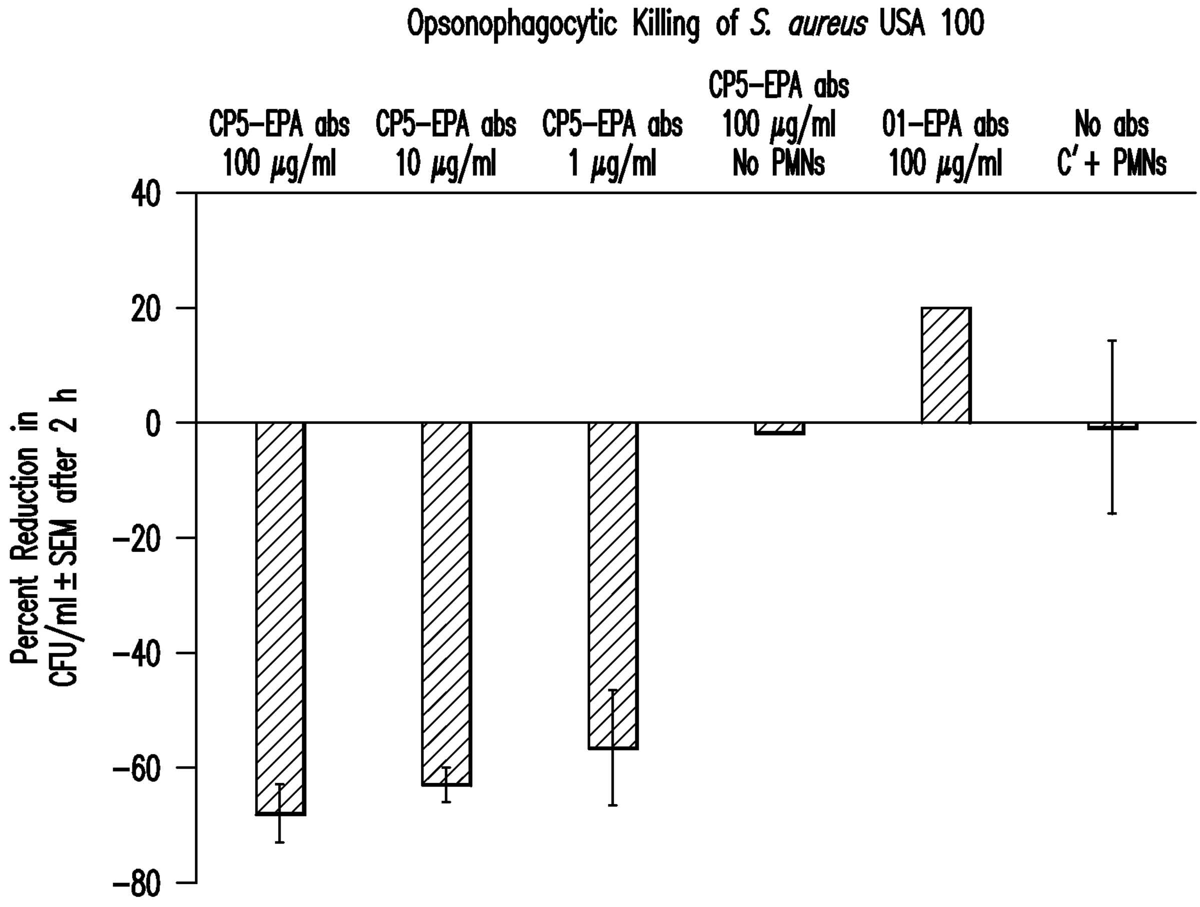


FIG.16B

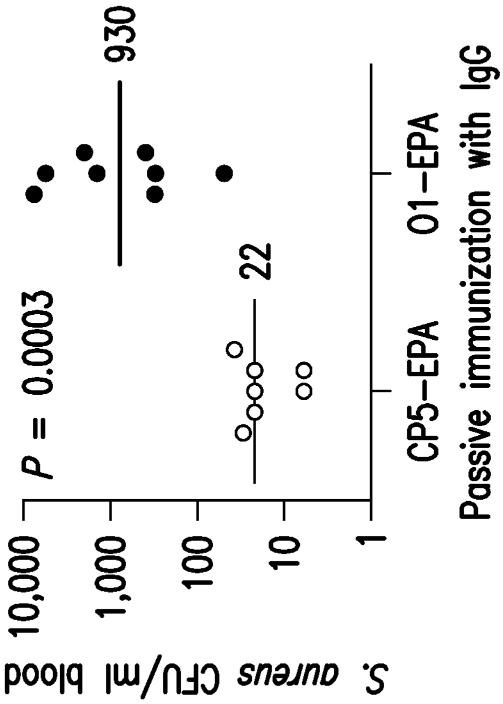


FIG.17B

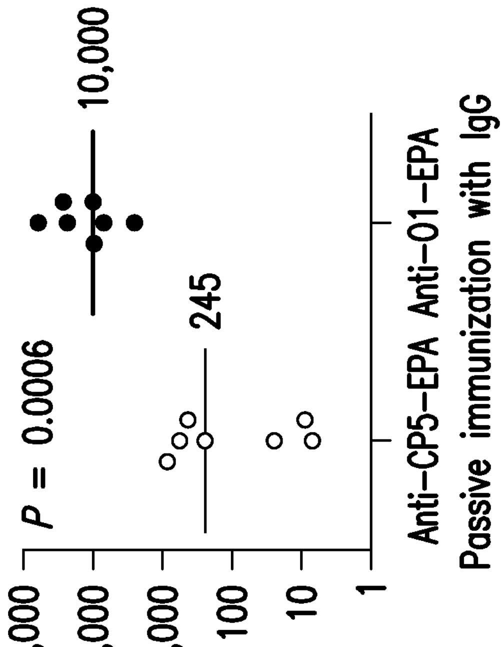


FIG.17A

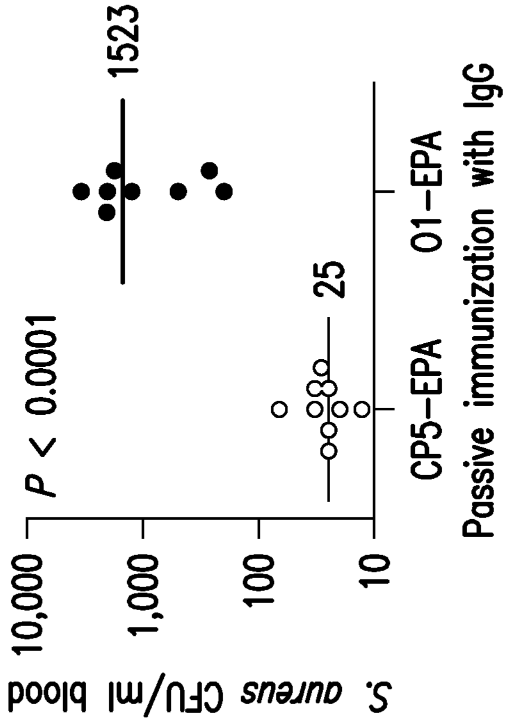


FIG.17C

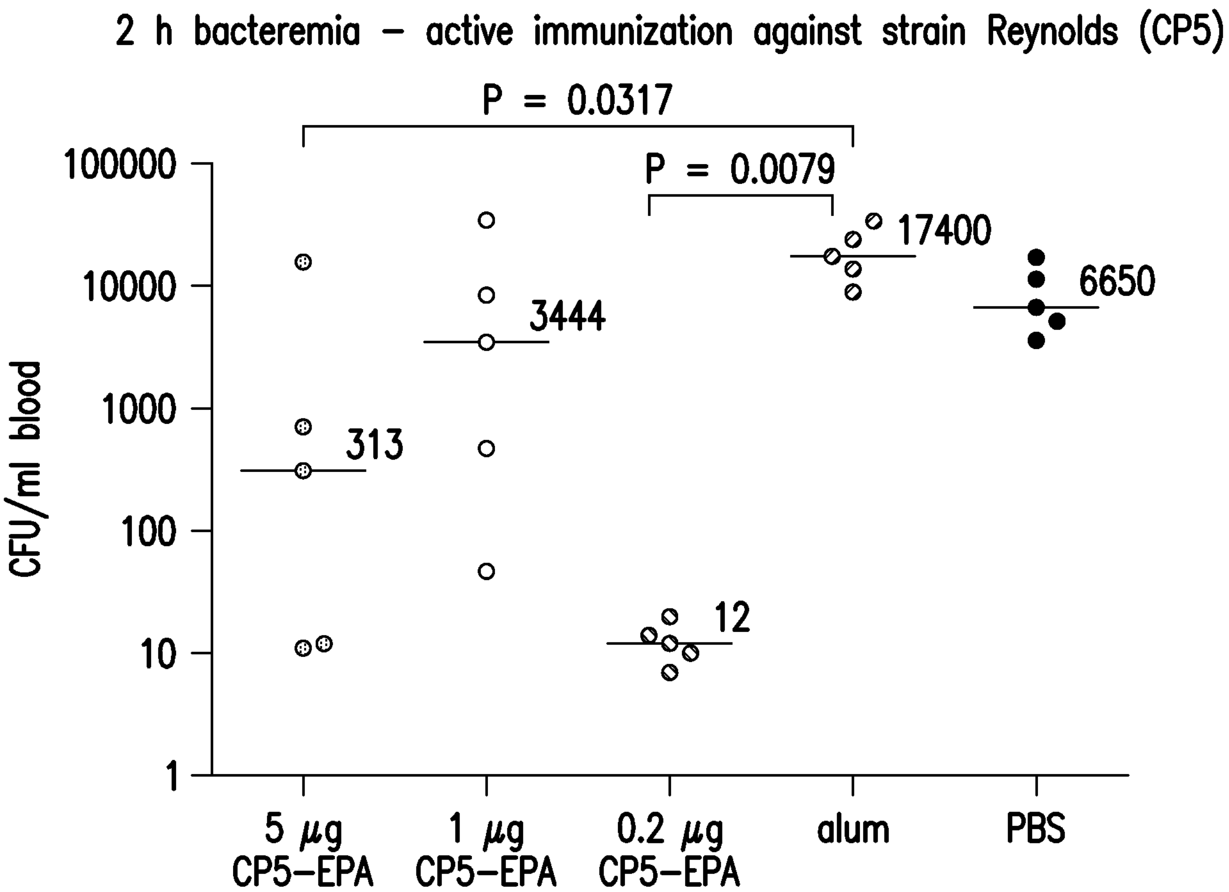
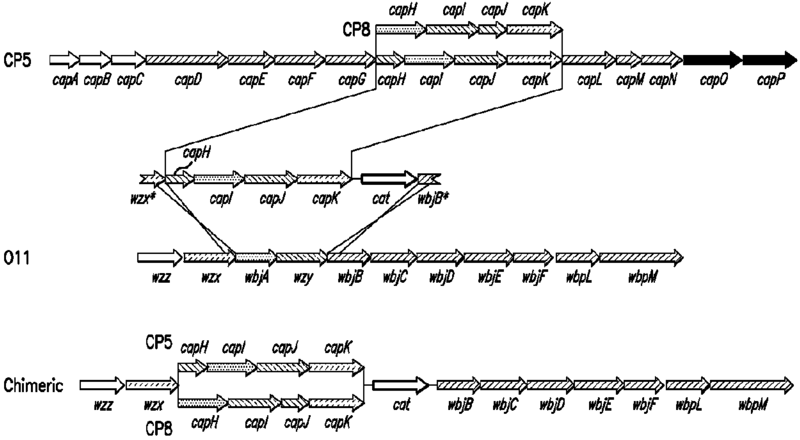

2 h bacteremia – active immunization against strain Reynolds (CP5)
P = 0.0317
P = 0.0079
100000
10000
1000
100
10
1
CFU/ml blood
17400
6650
3444
313
12
5 μg CP5-EPA
1 μg CP5-EPA
0.2 μg CP5-EPA
alum
PBS

FIG.18


CP5
capA
capB
capC
capD
capE
capF
capG
capH
capI
capJ
capK
capL
capM
capN
capO
capP
CP8
capH
capI
capJ
capK
wzx*
capH
capI
capJ
capK
cat
wbjB*
O11
wzz
wzx
wbjA
wzy
wbjB
wbjC
wbjD
wbjE
wbjF
wbpL
wbpM
Chimeric
CP5
capH
capI
capJ
capK
CP8
capH
capI
capJ
capK
wzz
wzx
cat
wbjB
wbjC
wbjD
wbjE
wbjF
wbpL
wbpM