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DESCRIPTION

1. INTRODUCTION

[0001] Described herein are oligosaccharyl transferases for use in N-glycosylating proteins of interest *in vitro* and in host cells. Methods for using such oligosaccharyl transferases, nucleic acids encoding such oligosaccharyl transferases, and host cells comprising such oligosaccharyl transferases are also provided herein. Glycoconjugates generated by using such oligosaccharyl transferases are also provided herein.

2. BACKGROUND

[0002] Glycoconjugate vaccines are widely recognized for their ability to prevent many lifethreatening bacterial infections. Glycoconjugate vaccines are generally considered efficacious and safe and have been used in humans for over 30 years. Conventional glycovaccine production often involves the chemical modification of immunogenic carrier proteins with polysaccharide antigens of pathogenic bacteria. However, more recently, biotechnological processes for producing glycoconjugate vaccines have emerged that are expected to reduce production costs and to further increase the homogeneity and possibly the potency and safety of glycoconjugate vaccine preparations.

[0003] In eukaryotic cells, N-linked glycosylation is a key posttranslational protein modification mechanism involving several enzymes. In prokaryotic cells N-linked glycosylation is catalyzed by certain bacterial N-oligosaccharyltransferases (N-OSTs). The protein glycosylation gene cluster of *Campylobacter jejuni (C. jejuni)* includes the *pglB* gene, which encodes a membrane-bound N-OST ($PglB_{Cj}$). $PglB_{Cj}$ can be expressed in standard bacterial hosts, such as *Escherichia coli (E. coli),* and can glycosylate co-expressed periplasmic proteins that carry at least one surface-exposed D/E-Y-N-X-S/T. (Y, X ≠ P) glycosylation motif. $PglB_{Cj}$ can transfer bacterial polysaccharide antigens to *C. jejuni* proteins as well as to immunogenic carrier proteins of other organisms containing engineered glycosylation sites. $PglB_{Cj}$ can transfer *C. jejuni* oligosaccharides and, to a certain degree, O-antigen lipopolysaccharide structures of Gram-negative bacteria and capsular antigen polysaccharides of Gram-positive bacteria. Lizak et al in Journal of Biological Chemistry vol. 289, no 2, pages 735-746 (2013) describes a catalytically essential motif in external loop 5 of the bacterial oligosaccharyltransferase PglB.

[0004] The present disclosure provides recombinant N-OSTs with modified substrate specificities and methods of using the recombinant N-OSTs for glycoconjugate vaccine production. Such recombinant N-OSTs can advantageously be used in N-glycosylation of proteins.

3. SUMMARY

[0005] In one aspect, provided herein is a recombinant N-oligosaccharyl transferase which is is PglB of *Campylobacter jejuni* (PglB_{Cj}) or PglB of *Campylobacter lari* (PglB_{Cl}), wherein the recombinant N-oligosaccharyl transferase (N-OST) can detectably link an oligosaccharide or polysaccharide lacking an N-acetyl sugar at the reducing end to a carrier protein at an N-glycosylation consensus sequence wherein one or more amino acids selected from the group consisting of Y77, S80, S196, N311, Y462, K482, D483 and G477 of PglB_{Cj} are modified.

[0006] In some embodiments, the N-OST activity of linking the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end to the carrier protein at the N-glycosylation sequence is detected by ELISA.

[0007] In some embodiments, the ELISA signal indicating the N-OST activity is detectable if it is $>2\sigma$ or $>3\sigma$ above the ELISA background signal.

[0008] In some embodiments, the carrier protein is a natural carrier protein from the same organism as the N-OST. In some embodiments, the carrier protein is a heterologous carrier protein from a different organism than the N-OST.

[0009] In some embodiments, the carrier protein is selected from the group consisting of exotoxin A of *P. aeruginosa* (EPA), CRM197, diphtheria toxoid, tetanus toxoid, detoxified hemolysin A of *S. aureus,* clumping factor A, clumping factor B, *E. coli* FimH, *E. coli* FimHC, *E. coli* heat labile enterotoxin, detoxified variants *of E. coli* heat labile enterotoxin, Cholera toxin B subunit (CTB), cholera toxin, detoxified variants of cholera toxin, *E. coli* sat protein, the passenger domain *of E. coli* sat protein, *C. jejuni* AcrA, and *C. jejuni* natural glycoproteins.

[0010] In some embodiments, the carrier protein has at least one glycosylation motif. In some embodiments, the at least one glycosylation motif comprises D/E-Y-N-X-S/T (X, Y ≠ P). In some embodiments, the at least one glycosylation motif comprises Asn-X-Ser(Thr), wherein X can be any amino acid except Pro. In some embodiments, the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end comprises an antigen.

[0011] In some embodiments, the antigen includes an *E. coli* antigen, a *Salmonella* sp antigen, a *Pseudomonas* sp. antigen, a *Klebsiella* sp. antigen, a acinetobacter O antigen, a *Chlamydia trachomatis* antigen, a *Vibrio cholera* antigen, a *Listeria* sp. antigen, a *Legionella pneumophila* serotypes 1 to 15 antigen, a *Bordetella parapertussis* antigen, a *Burkholderia mallei* or *pseudomallei* antigen, a *Francisella tularensis* antigen, a *Campylobacter* sp. antigen; a *Clostridium difficile* antigen, *Streptococcus pyrogenes* antigen, a *Streptococcus agalacticae* antigen, a *Neisseria meningitidis* antigen, a *Candida albicans* antigen, a *Haemophilus influenza* antigen, a *Enterococcus faecalis* antigen, a *Borrelia burgdorferi* antigen, a *Neisseria meningitidis* antigen, a *Haemophilus influenza* antigen, a *Leishmania major* antigen, or a *Shigella sonnei,* or *Streptococcus pneumoniae* antigen *(e.g.,* CP1, CP4, and the like).

[0012] In some embodiments, the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end is a *Staphylococcus aureus* or a *Salmonella enterica sv.* polysaccharide. In some embodiments, the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end is a *Staphylococcus aureus* CP5 or a *Salmonella enterica sv.* Typhimurium LT2 polysaccharide.

[0013] In some embodiments, the recombinant N-oligsaccharyl transferase can increase the yield of *in vivo* glycosylation or *in vitro* glycosylation of the carrier protein with the polysaccharide lacking the N-acetyl sugar at the reducing end to produce glycosylated carrier protein at a level of more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold above background level in an assay detecting the glycosylated carrier protein.

[0014] In some embodiments, the recombinant N-oligsaccharyl transferase can increase the rate of *in vivo* glycosylation or *in vitro* glycosylation of the carrier protein with the polysaccharide lacking the N-acetyl sugar at the reducing end by more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold compared to a wild-type form of the recombinant N-oligosaccharyl transferase.

[0015] In some embodiments, the recombinant N-oligosaccharyl transferase can *in vivo* or *in vitro* glycosylate at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of the carrier protein with the polysaccharide lacking the N-acetyl sugar at the reducing end.

[0016] In some embodiments, the recombinant N-oligosaccharyl transferase comprises a modification in one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-oligosaccharyl transferase and the N-glycosylated carrier protein.

[0017] In some embodiments, the 2.5-4.0 Ådistance is the distance from the first terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Ådistance is from the second terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Ådistance is from the third terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Ådistance is from a conserved amino acid in the catalytic center of the recombinant N-oligosaccharyl transferase in the structural model of a complex of the recombinant N-oligosaccharyl transferase and the N-glycosylated carrier protein (*e.g.*, K522, N311, H 479, G476, Y462, G477, Y77, S80, or S199 of $PglB_{Cj}$), *see, e.g.,* FIG.2).

[0018] In some embodiments, the modification in the one or more amino acids is an amino acid substitution.

[0019] In some embodiments, the one or more amino acids include an amino acid that is a non-conserved amino acid in a phylogenetic family of N-oligosaccharyl transferases. In some embodiments, the non-conserved amino acid is conserved in less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20% or less than 10% of members of the phylogenetic family of N-oligosaccharyl transferases.

[0020] In some embodiments, the recombinant N-oligosaccharyl transferase comprises a modification in two or more amino acids. In some embodiments, the recombinant N-oligosaccharyl transferase comprises modification in three or more amino acids. In some embodiments, the recombinant N-oligosaccharyl transferase comprises modification in four or more amino acids.

[0021] In some embodiments, at least one of the one or more amino acids is located in a periplasmatic loop of a transmembrane domain of the recombinant N-oligosaccharyl transferase. In some embodiments, the periplasmatic loop of the transmembrane domain is a large external loop 5 (EL5). In some embodiments, the recombinant N-oligosaccharyl transferase is $PglB_{Cj}$ and the EL5 is EL5 of $PglB_{Cj}$.

[0022] In some embodiments, the recombinant N-oligosaccharyl transferase further comprises a mutation in one or more amino acids in a QLKFYxxR motif. In some embodiments, the Q287LKFYxxR294 motif is a Q287LKFYxxR294 motif. In some embodiments, the Q287LKFYxxR294 motif is the Q287LKFYxxR294 motif of $PglB_{Cj}$.

[0023] In some embodiments, the recombinant N-oligosaccharyl transferase is a recombinant $PglB_{Cj}$.

[0024] In some embodiments, the bound N-glycosylated carrier protein is a natural *C. jejuni* glycosylated carrier protein. In some embodiments, the bound N-glycosylated carrier protein is a heterologous *C. jejuni* glycosylated carrier protein.

[0025] In some embodiments, the oligosaccharide or polysaccharide component of the bound N-glycosylated carrier protein has a galactose monosaccharide at its reducing end.

[0026] In some embodiments, one or more amino acids selected from the group consisting of Y77, S80, S196, N311, Y462, H479, K522, G476 and G477 of PglB$_{Cj}$ are modified. In some embodiments, N311 of PglB$_{Cj}$ is modified. In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution N311V or a substitution N311I. In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution N311V. In some embodiments, the recombinant PglB$_{Cj}$ further comprises a modification in one or more amino acids selected from the group consisting of Y77 and S80. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitution selected from the group consisting of Y77H, Y77T, Y77W, Y77R, Y77K, Y77A, Y77G, S80R and S80H. In some embodimens, the recombinant PglB$_{Cj}$ comprises an amino acid substitution selected from the group consisting of Y77H and S80R.

[0027] In some embodiments, the recombinant PglB$_{Cj}$ further comprises an amino acid modification in one or more amino acids of the Q287LKFYxxR294 motif of PglB$_{Cj}$. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid modification in one or more amino acids selected from the group consisting of Q287, L288 and K289. In some embodiments, the recombinant PglB$_{Cj}$ comprises one or more amino acid substitutions selected from the group consisting of Q287P, Q287K, Q287R, L288M, L288F, L288I, L288C, K289R, K289N, K289Q and R294K.

[0028] In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitution N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions Y77H and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions S80R and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions Q287P and Y77H or a Q287P and S80R. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions S80R, Q287P and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions Y77H, Q287P and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions Y77H, S80R, Q287P and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions Y77H, S80R, Q287P, K289R and N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions N311V and A699V. In some embodiments, the recombinant PglB$_{Cj}$ comprises amino acid substitutions K482R and D483H.

[0029] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase (N-OST) comprising a modification in one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-oligosaccharyl transferase and the N-glycosylated carrier protein. In some embodiments, the modification is an amino acid substitution.

[0030] In some embodiments, the carrier protein is selected from the group consisting of exotoxin A of *P. aeruginosa* (EPA), CRM197, diphtheria toxoid, tetanus toxoid, detoxified hemolysin A of *S. aureus,* clumping factor A, clumping factor B, *E. coli* FimH, *E. coli* FimHC, *E. coli* heat labile enterotoxin, detoxified variants *of E. coli* heat labile enterotoxin, Cholera toxin B subunit (CTB), cholera toxin, detoxified variants of cholera toxin, *E. coli* sat protein, the passenger domain of *E. coli* sat protein, *C. jejuni* AcrA, and *C. jejuni* natural glycoproteins.

[0031] In some embodiments, the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end comprises an antigen. In some embodiments, the antigen includes an *E. coli* antigen, a *Salmonella* sp antigen, a *Pseudomonas* sp. antigen, a *Klebsiella* sp. antigen, an acinetobacter O antigen, a *Chlamydia trachomatis* antigen, a *Vibrio cholera* antigen, a *Listeria* sp. antigen, a *Legionella pneumophila* serotypes 1 to 15 antigen, a *Bordetella parapertussis* antigen, a *Burkholderia mallei* or *pseudomallei* antigen, a *Francisella tularensis* antigen, a *Campylobacter* sp. antigen; a *Clostridium difficile* antigen, a *Streptococcus agalacticae* antigen, a a *Neisseria meningitidis* antigen, a *Candida albicans* antigen, a *Haemophilus influenza* antigen, a *Enterococcus faecalis* antigen, a *Borrelia burgdorferi* antigen, a *Neisseria meningitidis* antigen, a, *Haemophilus influenza* antigen, a *Leishmania major* antigen, a Shigella *sonnei,* or a Streptococcus pneumoniae antigen *(e.g.,* CP1, CP4, and the like).

[0032] In some embodiments, the recombinant N-oligosaccharyl transferase comprises modifications in two or more amino acids. In some embodiments, the recombinant N-oligosaccharyl transferase comprises modifications in three or more amino acids. In some embodiments, the recombinant N-oligosaccharyl transferase comprises modifications in four or more amino acids.

[0033] In some embodiments, at least one of the one or more amino acids is located in a periplasmatic loop of a transmembrane domain of the recombinant N-oligosaccharyl transferase. In some embodiments, the periplasmatic loop of the transmembrane domain is a large external loop 5 (EL5). In some embodiments, the recombinant N-oligosaccharyl transferase is PglB of *Campylobacter jejuni* (PglB$_{Cj}$) and EL5 is EL5 of PglB$_{Cj}$.

[0034] In some embodiments, the recombinant N-oligosaccharyl transferase further comprises a modification in one or more amino acids in a QLKFYxxR motif. In some embodiments, wherein the recombinant N-oligosaccharyl transferase further comprises a modification in one or more amino acids in a Q287LKFYxxR294 motif. In some embodiments, the QLKFYxxR motif is the Q287LKFYxxR294 motif of PglB$_{Cj}$.

[0035] In some embodiments, the amino acid substitution is a substitution of a non-conserved amino acid in a phylogenetic family of N-oligosaccharyl transferases.

[0036] In some embodiments, the bound N-glycosylated polypeptide product is a natural N-glycosylated carrier protein from the same organism as the recombinant N-oligosaccharyl transferase. In some embodiments, the N-glycosylated carrier protein is a heterologous N-glycosylated carrier protein, wherein the oligosaccharide or polysaccharide component of the

N-glycosylated carrier protein is from a different organism than the recombinant N-oligosaccharyl transferase and/or the carrier protein component of the N-glycosylated carrier protein is from a different organism than the recombinant N-oligosaccharyl transferase.

[0037] In some embodiments, the recombinant N-oligosaccharyl transferase is recombinant PglB$_{Cj}$.

[0038] In some embodiments, the bound N-glycosylated polypeptide product is a natural *C. jejuni* glycosylated carrier protein. In some embodiments, the bound N-glycosylated polypeptide product is a heterologous *C. jejuni* glycosylated carrier protein. In some embodiments the heterologous *C. jejuni* glycosylated carrier protein is *Pseudomonas aeruginosa* exotoxin (EPA)-*S. dysenteriae* O1 (EPA-O1), EPA-*S. aureus* capsular polysaccharide Type 5 (EPA-CP5) or EPA-*Salmonella enterica (S. enterica)* LT2 (EPA-LT2).

[0039] In some embodiments, the oligosaccharide or polysaccharide component of the bound N-glycosylated carrier protein does not have an N-acetyl monosaccharide at its reducing end. In some embodiments, the oligosaccharide or polysaccharide component of the bound N-glycosylated carrier protein has a galactose monosaccharide at its reducing end.

[0040] In some embodiments, one or more amino acids from the group consisting of Y77, S80, S196, N311, Y462, H479, K522, G476 and G477 of PglB$_{Cj}$ are modified. In some embodiments, of PglB$_{Cj}$ is modified. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitution selected from the group consisting of N311V and N311I. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitution N311V. In some embodiments, one or more amino acids selected from the group consisting of Y77 and S80 of PglB$_{Cj}$ or modified. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitution selected from the group consisting of Y77H, Y77T, Y77W, Y77R, Y77K, Y77A, Y77G, S80R and S80H. In some embodiments, the recombinant PglB$_{Cj}$ comprises an amino acid substitutions selected from the group consisting of Y77H and S80R.

[0041] In some embodiments, the recombinant PglB$_{Cj}$ further comprises a modification of one or more amino acids of the Q287LKFYxxR294 motif of PglB$_{Cj}$. In some embodiments, the recombinant PglB$_{Cj}$ comprises a modification of one more amino acids selected from the group consisting of Q287, L288 and K289. In some embodiments,the recombinant PglB$_{Cj}$ comprises a substitution selected from the group consisting of Q287P, Q287K, Q287R, L288M, L288F, L288I, L288C, K289R, K289N, K289Q and R294K.

[0042] In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution Y77H and a substitution N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution S80R and a substitution N311V. In some embodiments, the recombinant PglB$_{Cj}$ comprises a substitution Q287P and a substitution Y77H or a substitution Q287P mutation and a substitution S80R. In some embodiments, the recombinant PglB_{Cj} comprises a substitution S80R, a substituion Q287P and a substitution N311V. In some embodiments, the recombinant PglB_{Cj} comprises a substitution Y77H, a substitution Q287P and a substitution N311V. In some embodiments, the recombinant PglB_{Cj} comprises a substitution Y77H, a substitution S80R, a substitution Q287P and a substitution N311V. In some embodiments, the recombinant PglB_{Cj} comprises a substitution Y77H, a substitution S80R, a substitution Q287P, a substitution K289R and a substitution N311V. In some embodiments, the recombinant PglB_{Cj} comprises a substitution N311V and a substitution A699V. In some embodiments,the recombinant PglB_{Cj} comprises a substitution K482R, and a substitution D483H.

[0043] In some embodiments, the recombinant N-oligosaccharyl transferase can detectably link an oligosaccharide or polysaccharide lacking an N-acetyl sugar at the reducing end to a carrier protein.

[0044] In some embodiments, the recombinant N-oligosaccharyl transferase can detectably link an oligosaccharide or polysaccharide having a galactose monosaccharide at the reducing end to a carrier protein.

[0045] In some embodiments, the oligosaccharide or polysaccharide is a *Staphylococcus aureus* or a *Salmonella enterica sv.* oligosaccharide or polysaccharide. In some embodiments, the oligosaccharide or polysaccharide is a *Staphylococcus aureus* CP5 or a *Salmonella enterica* sv. Typhimurium LT2 oligosaccharide or polysaccharide.

[0046] In some embodiments, the recombinant N-oligsaccharyl transferase can increase the yield of *in vivo* glycosylation or *in vitro* glycosylation of the carrier protein with the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end to produce glycosylated carrier protein at a level of more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold above background level in an assay detecting the glycosylated carrier protein.

[0047] In some embodiments, the recombinant N-oligsaccharyl transferase can increase the *in vivo* or *in vitro* rate of glycosylation of a carrier protein with the oligosaccharide or polysaccharide lacking the N-acetyl sugar at the reducing end by more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold compared to a wild-type form of the recombinant N-oligosaccharyl transferase.

[0048] In some embodiments, the recombinant N-oligosaccharyl transferase can yield an *in vivo* glycosylation level or an *in vitro* glycosylation level of the carrier protein of at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, or at least 70%.

[0049] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V substitution.

[0050] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation and a Y77H substitution.

[0051] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation and a S80R substitution.

[0052] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation and a Y77H mutation and a S80R substitution.

[0053] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation and a Q287P substitution.

[0054] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation, a Y77H substitution and a Q287P substitution.

[0055] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V mutation, S80R substitution and a Q287P substitution.

[0056] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V substitution, a Y77H substitution, a S80R substitution and a Q287P substitution.

[0057] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V substitution and a A669V substitution.

[0058] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V substitution, a Y77H substitution, a S80R substitution, a Q287P substitution and a K289R substitution.

[0059] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a K482R substitution and a D483H substitution.

[0060] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase $PglB_{Cj}$ comprising a N311V substitution and a A669V substitution.

[0061] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ (PglB *C. lari)* comprising a N314V substitution.

[0062] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation and a Y79H substitution.

[0063] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation and a S82R substitution.

[0064] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation and a Y79H mutation and a S82R substitution.

[0065] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation and a Q289P substitution.

[0066] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation, a Y79H substitution and a Q289P substitution.

[0067] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V mutation, S82R substitution and a Q289P substitution.

[0068] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a N314V substitution, a Y79H substitution, a S82R substitution and a Q289P substitution.

[0069] In another aspect, provided herein is a recombinant N-oligosaccharyl transferase PglB$_{Cl}$ comprising a K488R substitution and a D489H substitution.

[0070] In another aspect, provided herein is a nucleic acid encoding a recombinant N-oligosaccharyl transferase described herein.

[0071] In another aspect, provided herein is a host cell comprising a recombinant N-oligosaccharyl transferase described herein.

[0072] In some embodiments, the host cell further comprises a recombinant glycosyltransferase.

[0073] In another aspect, provided herein is a host cell comprising a nucleic acid described herein.

[0074] In some embodiments, the host cell is a prokaryotic cell. In some embodiments, the host cell is an *E. coli* cell.

[0075] In another aspect, provided herein is a method of producing a bioconjugate comprising culturing a host cell described herein.

[0076] In some embodiments, the host cell comprises a carrier protein and a recombinant N-oligosaccharyl transferase. In some embodiments, the host cell further comprises a recombinant glycosyltransferase. In some embodiments, the recombinant N-oligosaccharyl transferase is a recombinant $PglB_{Cj}$.

[0077] In some embodiments, the carrier protein is selected from the group consisting of exotoxin A of *P. aeruginosa* (EPA), CRM197, diphtheria toxoid, tetanus toxoid, detoxified hemolysin A of *S. aureus,* clumping factor A, clumping factor B, *E. coli* FimH, *E. coli* FimHC, *E. coli* heat labile enterotoxin, detoxified variants *of E. coli* heat labile enterotoxin, Cholera toxin B subunit (CTB), cholera toxin, detoxified variants of cholera toxin, *E. coli* sat protein, the passenger domain *of E. coli* sat protein, *C. jejuni* AcrA, and *C. jejuni* natural glycoproteins.

[0078] In some embodiments, the bioconjugate is an N-glycosylated carrier protein. In some embodiments, the bioconjugate is a natural *C. jejuni N*-glycosylated carrier protein. In some embodiments, the bioconjugate is a heterologous *C. jejuni N*-glycosylated carrier protein. In some embodiments, the N-glycosylated carrier protein does not have an N-acetyl sugar at the reducing end of its oligosaccharide or polysaccharide component. In some embodiments, the N-glycosylated carrier protein has a galactose at the reducing end of its oligosaccharide or polysaccharide component.

[0079] In some embodiments, the recombinant N-oligosaccharyl transferase mutant can increase the rate of bioconjugate production by more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold compared to the rate achieved with a wild-type form of the recombinant N-oligosaccharyl transferase.

[0080] In some embodiments, the recombinant N-oligsaccharyl transferase mutant can increase the yield of bioconjugate production to a level of more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold the above background level in an assay detecting the bioconjugate.

[0081] In some embodiments, at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of carrier protein in a host cell is glycosylated to form the bioconjugate.

[0082] In some embodiments, the method further comprises purifying the bioconjugate from the host cell culture.

[0083] In another aspect, provided herein is a method of screening a library of recombinant N-oligosaccharyl transferases each recombinant N-oligosaccharyl transferase comprising a modification in one or more amino acids, comprising contacting each member of the library of recombinant N-oligosaccharyl transferases with a carrier protein and an oligosaccharide or polysaccharide lacking an N-acetyl sugar at its reducing end to produce a bioconjugate.

[0084] In some embodiments, the bioconjugate is an N-glycosylated carrier protein.

[0085] In some embodiments, the contacting occurs *in vitro.* In some embodiments, the contacting occurs *in vivo.* In some embodiments, the contacting occurs in a host cell. In some embodiments, the host cell is a prokaryotic cell. In some embodiments, the host cell is an *E. coli* cell.

[0086] In some embodiments, the library of recombinant N-oligosaccharyl transferases comprises at least 2, at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 500, at least 750 or at least 1,000 recombinant N-oligosaccharyl transferases.

[0087] In some embodiments, the library of recombinant N-oligosaccharide transferases comprises one or more recombinant N-oligosaccharide transferases described herein.

[0088] In some embodiments, the method further comprises analyzing the rate or yield of production of the bioconjugate.

[0089] In some embodiments, the method further comprises selecting one or more recombinant N-oligosaccharyl transferases from the library of recombinant N-oligosaccharyl transferases.

[0090] In some embodiments, the one or more recombinant N-oligosaccharyl transferase is selected if the recombinant N-oligosaccharyl transferase yields the bioconjugate at a rate that is more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold faster than the rate of a wild-type form of the recombinant N-oligosaccharyl transferase.

[0091] In some embodiments, the one or more N-oligosaccharyl transferase mutant is selected if the N-oligosaccharyl transferase mutant yields the bioconjugate at a yield that is detectable at a level of more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold above background level in an assay detecting the bioconjugate.

[0092] In some embodiments, the one or more recombinant N-oligosaccharyl transferase is selected if the recombinant N-oligosaccharyl transferase glycosylates at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of a carrier protein in the host cell.

[0093] In another aspect, provided herein is a method of identifying a recombinant N-oligosaccharyl transferase having a modified substrate selectivity relative to a wild-type form of the N-oligosaccharyl transferase, comprising modifying one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-oligosaccharyl transferase and the N-glycosylated carrier protein.

[0094] In some embodiments, the method comprises modifying two or more amino acids of the recombinant N-oligosaccharyl transferase. In some embodiments, the method comprises modifying three or more amino acids of the recombinant N-oligosaccharyl transferase. In some embodiments, the method comprises modifying four or more amino acids of the recombinant N-oligosaccharyl transferase.

[0095] In some embodiments, at least one of the one or more amino acids is located in a periplasmatic loop of a transmembrane domain of the recombinant N-oligosaccharyl transferase. In some embodiments, the periplasmatic loop of the transmembrane domain is a large external loop 5 (EL5).

[0096] In some embodiments, the method further comprises mutating one or more amino acids in a QLKFYxxR motif of the recombinant N-oligosaccharyl transferase. In some embodiments, the QLKFYxxR motif is a Q287LKFYxxR294 motif. In some embodiments, the bound N-glycosylated carrier protein is a natural N-glycosylated carrier protein.

[0097] In some embodiments, the bound N-glycosylated carrier protein is a heterologous N-glycosylated carrier protein. In some embodiments, the recombinant N-oligosaccharyl transferase is a recombinant $PglB_{Cj}$. In some embodiments, the bound N-glycosylated carrier protein is a natural *C. jejuni* N-glycosylated carrier protein. In some embodiments, the bound N-glycosylated carrier protein is a heterologous *C. jejuni* N-glycosylated carrier protein.

[0098] In some embodiments, the oligosaccharide or polysaccharide component of the bound N-glycosylated carrier protein does not have an N-acetyl monosaccharide at its reducing end. In some embodiments, the oligosaccharide or polysaccharide component of the bound N-glycosylated carrier protein has a galactose monosaccharide at its reducing end.

[0099] In some embodiments, the recombinant N-oligosaccharyl transferase has a modified substrate selectivity *in vitro.* In some embodiments, the recombinant N-oligosaccharyl transferase has a modified substrate selectivity *in vivo.*

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0100]

FIG. 1. depicts structures of the natural *C. jejuni* heptasaccharide substrate of PglB$_{Cj}$ and of two non-natural polysaccharide substrates with decreasing glycosylation efficiency from top to bottom. GalNAc: 2-N-acetylgalactosamine, Glc: glucose, DATDH: 2,4-diacetamido-2,4,6-trideoxyhexose, P-P-und: undecaprenyl-pyrophosphate carrier, Rha: rhamnose, Gal: galactose, GlcNAc: N-acetylglucosamine, ManNAc: N-acetylmannosamine, OAc: O-acetyl modification, FucNAc: N-acetylfucosamine, Man: mannose, Abe: abequose (3,6-deoxy-D-galactose).

FIG. 2 depicts an exemplary modeling of oligosaccharide structures when interacting with PglB$_{Cj}$.FIG. 2A depicts an exemplary conformation of *C. jejuni* OS (light grey ball-and-stick representation) and *S. enterica* LT2 repeating unit (dark grey stick representation) in the active site, the position of the linkage to the acceptor peptide was chosen as fix point during dynamic modeling. FIG. 2B depicts an exemplary conformation of *C. jejuni* OS (light grey ball-and-stick representation). FIG. 2C depicts an exemplary conformation of *S. enterica* LT2 repeating unit (dark grey stick representation). The PglB$_{Cj}$ backbone structure is shown in grey (ribbon) and the phosphate groups of the membrane as light grey balls. Residues in close proximity to the natural OS are depicted as light grey ball-stick representations. A broken line illustrates the connectivity of the unstructured external loop EL5.

FIG. 3 illustrates results of an exemplary DWP-ELISA screening of a saturation mutagenesis library randomizing PglB$_{Cj}$ residue N311. FIG. 3A depicts screening results using host strain and detection antibodies for *S. aureus* CP5 polysaccharides. FIG. 3B depicts screening results using host strain and detection antibodies for *S. enterica* LT2 polysaccharides. Open circles indicate library clones; filled triangles indicate positive control clones expressing wild-type PglB (pGVXN1413), shaded triangles indicate negative control clones expressing inactive PglB$_{mut}$ (pGVXN408). Sequenced clones are marked by an ellipsoid.

FIG. 4 depicts alignments of bacterial PglB homologues (A) in the EL5 region, including the *C. jejuni* 287QLKFYxxR294 motif and *C. jejuni* N311, and (B) in the vicinity of residues *C. jejuni* Y77/S80 and *C. jejuni* K482/D843. PglB of *C. jejuni* was used as search template for Protein BLAST and non-redundant sequences were aligned with the MegAlign™ program using the ClustalW algorithm (DNASTAR, Madison, WI, USA). PglB_{Cj} residues conserved in sequences of other species are shaded. Relevant *C. jejuni* residues are indicated at the top and corresponding amino acids in homologous N-OST sequences are boxed.

FIG. 5 depicts a graph illustrating the effect of amino acid substitutions within potential sugar-interacting PglB_{Cj} residues Y462, G476, G477 and H479 on *in vivo* CP5-EPA production in overnight induced DWP cultures. Reference wells (100% values, background corrected): pGVXN1050 (wild-type template plasmid). Average numbers and standard deviations of triplicate clones per variant are depicted.

FIG. 6 illustrates the effect of PglB variant N311V on glycoprotein formation in shake flask analyzed by Western blot. FIG. 6A illustrates results obtained with LT2-EPA in host strain *S. enterica* SGSC228 (pGVXN150). FIG. 6B illustrates results obtained with CP5-EPA in host strain *E. coli* St1717 (pGVXN150, pGVXN393). FIG. 6C illustrates results obtained with O1-EPA in host strain *E. coli* CLM24 (pGVXN64, pGVXN150). FIG. 6D illustrates results obtained with EPA-*C. jejuni* OS in host strain *E. coli* CLM24 (pACYC(pgl_{mut}), pGVXN150). Same experiments as shown in FIG. 5, biomass-normalized periplasmic extracts, similar loading volumes, samples of one shake flask culture per variant. Wild-type PglB: pGVXN970, PglB N311V: pGVXN1217. Theoretical molecular mass of unglycosylated EPA-6H: 69.4 kDa.

FIG. 7 illustrates the effect of amino acid substitution PglB_{Cj} N311V on glycosylation of EPA with three heterologous polysaccharides and natural oligosaccharides. Open symbols: wild-type PglB (pGVXN970), closed symbols; PglB N311V (pGVXN1217). FIG. 7A illustrates exemplary results obtained with the host strain and detection antibodies for *S. aureus* CP5 polysaccharides. FIG. 7B illustrates exemplary results obtained with the host strain and detection antibodies for *S. enterica* sv. Thyphimurium LT2 polysaccharides. FIG. 7C illustrates results obtained with the host strain and detection antibodies for *S. dysenteriae* O1 polysaccharides. FIG. 7D illustrates results obtained with the host strain and detection antibodies for *C. jejuni* oligosaccharides. Background-corrected ELISA signals for biomass-normalized periplasmic extracts from shake flask cultures, average values and standard deviations of n = 3 biological replicates are depicted.

FIG. 8 illustrates the effect of N311V on expression of HA-tagged PglB and CP5-EPA formation in a shake flask experiment. FIG. 8A illustrates results of an anti-HA Western blot analysis of PglB-HA in an *E. coli* St1717 (pGVXN150, pGVXN393) host strain. FIG. 8B illustrates results of a time course of CP5-EPA formation analyzed by sandwich ELISA of biomass normalized periplasmic extracts. Open symbols depict results for wild-type PglB-HA. Closed symbols depict results for PglB-HA N311V. Average values and standard deviations for n = 3 replicate cultures are shown.

FIG. 9 depicts exemplary results of a third round of directed evolution of PglB_{Cj}, employing shuffling of neutral and slightly beneficial mutations. FIG. 10A illustrates screening results for a representative 96-well library. Open circles illustrate library clones; filled triangles illustrate PglB N311V (template plasmid pGVXN1418); shaded triangles illustrate inactive PglB_{mut} (pGVXN408). FIG. 10B illustrates a verification of improvements in DWP after retransformation. Average values and standard deviations for n = 3 replicate clones/wells per variant plasmid, wt: pGVXN1413 and N311V: pGVXN1418 are depicted. FIG. 10C illustrates exemplary results of a SDS-PAGE and Western blot analysis of Ni-NTA affinity purified proteins produced with either wild-type PglB (pGVXN970), PglB N311V (pGVXN1217) or PglB S80R-Q287P-N311V (library clone 2B2) in shake flasks (similar loading volumes, total protein concentration (A_{280}) was adjusted to 2 mg mL^{-1}). Theoretical molecular weight of unglycosylated EPA-6H: 69.4 kDa.

FIG. 10 depicts exemplary screening results for a PglB_{Cj} library with randomized residues PglB_{Cj} K482 and D483. Open circles: library clones, closed triangles: wild-type PglB-HA (pGVXN407), open triangles: inactive PglB (pGVXN408). Clone Fa8_G10 harboring the double mutation K482R-D483H is marked by a circle

FIG. 11 shows a bar graph illustrating that PglB_{Cj} K482-D483H can improve production of CP5-EPA in shake flask cultures. Biomass-normalized periplasmic protein extracts were prepared 4 h after induction and after overnight (o/n) incubation and analysed by sandwich ELISA. Filled bars: wild-type PglBCj-HA (pGVXN1 14), open bars: PglBCj-HA K482R-D483H (pGVXN635). Average values and standard deviations of n = 3 replicate cultures. Background ELISA absorbance was subtracted (inactive PglB, pGVXN115).

FIG. 12 shows a Western Blot analysis illustrating that PglB_{Cj} K482-D483H can improve glycosylation of *S. aureus* Hla with *S. aureus* CP5 polysaccharides. SDS-PAGE and Western blot analysis of periplasmic proteins after HisTrap purification are shown. Similar volumes of the elution fractions with the highest protein concentrations(A280) were loaded.

5. ABBREVIATIONS

[0101] The abbreviation "CP," as used herein, means "capsular polysaccharide."

[0102] The abbreviation "EL" as used herein, means "external loop."

[0103] The abbreviation "N-OST," as used herein, means N-oligosaccharyl transferase.

[0104] The abbreviation "PglB_{Cj}," as used herein, refers to the N-OST PglB of *Campylobacter jejuni (C. jejuni).*

[0105] The abbreviation "PglB_{Cl}," as used herein, refers to the N-OST PglB of *Campylobacter lari (C. lari).*

6. DETAILED DESCRIPTION

[0106] Provided herein is a modified N-oligosaccharyl transferase (N-OST) with a modified substrate specificity. Specifically, provided herein are modified N-OST's that are capable of using oligo- or polysaccharides as substrates for N-glycosylating proteins at an N-glycosylation consensus sequence that cannot be used (or cannot be used at detectable levels) by the wild type form of the N-OST. In certain embodiments, the modified N-OST can use such an oligo- or polysaccharide to produce detectable levels of an N-glycosylated carrier protein, *e.g., in vivo* or *in vitro.* Levels of glycosylated carrier protein can be determined by methods known in the art, including, without limitation, ELISA, HPLC, LC-MS and the like; *see, e.g.,* Section 6.10, Section 6.12, and Examples 2-3). In some embodiments, production of the N-glycoslated carrier protein is detected by ELISA.

[0107] In some embodiments, the levels of glycosylated carrier protein are detectable if the glycosylated carrier protein can be detected in an assay with a signal indicating glycosylated carrier protein at levels more than two or three standard deviations ($>2\sigma$ or $>3\sigma$) above the average or median assay background signal, or more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 7-fold, more than 8-fold, more than 9-fold, or more than 10-fold above the assay background signal.

[0108] In some embodiments, the assay background signal is the average or median assay signal of negative control experiments performed in the absence of an N-OST. In some embodiments, the assay background signal is the average or median assay signal of negative control experiments performed in the presence of a wild-type N-OST.

[0109] In certain embodiments, the glycosylated carrier protein can be detected at a level that is more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold above the background level in an assay detecting the bioconjugate *(e.g.,* in an ELISA assay, by HPLC, LC-MS; *see also* Section 6.10 and Section 6.12).

[0110] A modification in an N-OST provided herein can be located within a specified distance from the monosaccharide unit at the reducing end of the oligo- or polysaccharide component of a glycosylated carrier protein that is bound to the N-OST. To confirm that such a modification results in the altered substrate specificity any routine assay for protein glycosylation can be used. Such modified N-OST's can be used to generate bioconjugates in prokaryotic host cells as described herein. Compositions comprising the resulting bioconjugates are also disclosed herein. In a specific embodiment, such a modified N-OST is capable of using an oligo-or polysaccharide that lacks an N-acetyl substituted sugar at the reducing end as a substrate to produce detectable levels of a glycosylated carrier protein.

[0111] In some embodiments, the recombinant N-oligsaccharyl transferase mutant can increase the yield of bioconjugate production to a level of more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold above background level in an assay detecting the bioconjugate.

[0112] The background level in an assay detecting a bioconjugate can be, *e.g.,* the average or median signal obtained in control experiments that are performed in the absence of the N-OST or that are performed using wild-type N-OST.

6.1 N-Oligosaccharyl Transferases

[0113] In one aspect, provided herein is a recombinant N-oligosaccharyl transferase (N-OST), wherein the recombinant N-OST can detectably link an oligosaccharide or a polysaccharide lacking an N-acetyl sugar at the reducing end to a carrier protein. In some embodiments, the recombinant N-OST comprises modifications of one or more amino acids whose side chains are located within a 0.5-10.0 Å distance from the monosaccharide unit at the reducing end of the polysaccharide component of a bound glycosylated carrier protein product in a structural model of a complex of the recombinant N-OST and the glycosylated carrier protein product. In some embodiments, the modification is an amino acid substitution. In some embodiments, the recombinant N-OST comprises modifications of one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the polysaccharide component of a bound glycosylated carrier protein product in a structural model of a complex of the recombinant N-OST and the glycosylated carrier protein product. In some embodiments, the modification is an amino acid substitution. *See, e.g.,* FIG. 2 and Section 6.3.

[0114] In another aspect, provided herein is a recombinant N-OST including modifications of one or more amino acids whose side chains are located within a 1.0-10.0 Å distance from the monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein. In some embodiments, the recombinant N-OST is a recombinant N-OST including modifications of one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein. In some embodiments, the recombinant N-OST can detectably link an oligosaccharide or a polysaccharide lacking an N-acetyl sugar at the reducing end to a carrier protein. In some embodiments, the modification is an amino acid substitution. *See, e.g.,* FIG. 2 and Section 6.3.

[0115] In some embodiments, the 2.5-4.0 Å distance is the distance from the first terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Å distance is from the second terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Å distance is from the third terminal monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component. In some embodiments, the 2.5-4.0 Å distance is from a conserved amino acid in the catalytic center of the recombinant N-oligosaccharyl transferase in the structural model of a complex of the recombinant N-oligosaccharyl transferase and the N-glycosylated carrier protein (*e.g.*, K522, N311, H 479, G476, Y462, G477, Y77, S80, or S199 of PglB_{Cj}), *see, e.g.,* FIG.2).

[0116] In some embodiments, the 0.5-10.0 Å distance from the monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein is a distance of between about 1.0 and 9.0 Å, a distance of between about 1.5 and about 8.0 Å, a distance of between about 2.0 Å and about 6.0 Å or a distance of between about 2.5 Å and 4.0 Å. In some embodiments, the 1.0-10.0 Å distance from the monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein is a distance of about 1.0 Å, of about 1.5 Å, of about 2.0 Å, of about 2.5 Å, of about 3.0 Å, of about 3.5 Å, of about 4.0 Å, of about 4.5 Å, of about 5.0 Å, of about 5.5 Å, of about 6.0 Å, of about 6.5 Å, of about 7.0 Å, of about 7.5 Å, of about 8.0 Å, of about 8.5 Å, of about 9.0 Å or of about 10.0 Å. *See, e.g.,* FIG. 2 and Section 6.3.

[0117] Assays to confirm the activity of the N-OSTs described herein are well known to skilled artisans (e.g., ELISA, Western Blot) and include the assays described in Sections 6.10 and 6.12. In some embodiments, the recombinant N-OST includes modifications of one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein, the recombinant N-OST can detectably link the oligosaccharide or the polysaccharide lacking the N-acetyl sugar at the reducing end to the carrier protein and the activity of the N-OST can be confirmed by an assay described in Sections 6.10 or 6.12. In some embodiments, the modification is an amino acid substitution.

[0118] The oligosaccharides and polysaccharides can include any oligosaccharide or polysaccharide described herein. *See, e.g.,* Section 6.4.

[0119] The carrier proteins can comprise any carrier protein described herein. *See, e.g.,* Section 6.5.

[0120] In some embodiments, the recombinant N-OST comprises modifications in, *e.g.,* two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of an N-OST and the glycosylated carrier protein product.

[0121] In some embodiments, at least one or more modifications in the one more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of an N-OST and the glycosylated carrier protein product are located in a periplasmatic loop of a N-OST transmembrane domain. In some embodiments, the periplasmatic loop is the large external loop 5 (EL5) of an N-OST. In some embodiments, the periplasmatic loop is EL5 of PglB_{Cj}, of a PglB_{Cj} homologue, or of naturally occurring variants thereof *(see, e.g.,* FIGs. 4 and 9 for listing of PglB_{Cj} homologues).

[0122] N-OSTs can include conserved sequence motifs, such as the QLKFYxxR motif. *See, e.g.,* FIG.9. In some embodiments, the recombinant N-OST comprises modifications in at least one amino acids in the QLKFYxxR motif. In some embodiments, the QLKFYxxR motif is a Q287LKFYxxR294 motif *(see, e.g.,* PglB_{Cj} according to SEQ ID NO: 1). In some embodiments, the recombinant N-OST comprises modifications, *e.g.*, in at least two, at least three, at least four or at least five amino acids in the QLKFYxxRmotif. In some embodiments, the QLKFYxxR motif is the QLKFYxxR motif of PglB_{Cj}, of a PglB_{Cj} homologue, or of naturally occurring variants thereof.

[0123] In some embodiments, the recombinant N-OST comprises modifications of one or more amino acids whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of an N-OST and the N-glycosylated carrier protein and further comprises a modifications in one or more amino acids in a QLKFYxxR motif.

[0124] In some embodiments, the amino acid modifications comprise an amino acid substitution. An amino acid can be substituted for a natural proteinogenic amino acid or for an artificial amino acid. In some embodiments, the amino acid modifications comprise a substitution of a non-conserved amino acid (*i.e.,* modifications of amino acids that are not conserved between N-OSTs from different organisms). In some embodiments, the non-conserved amino acid is conserved in less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20% or less than 10% of members of the phylogenetic family of N-oligosaccharyl transferases. In some embodiments, the non-conserved amino acid is conserved in about between about 10% and about 90%, between about 20% and about 80%, between about 30% and about 70% or between about 40% and about 60% of members of the phylogenetic family of N-oligosaccharyl transferases.

[0125] In some embodiments, the recombinant N-OST can increase the *in vivo* or *in vitro* rate of glycosylation of a carrier protein with a polysaccharide by between about 2-fold and about 100-fold, by between about 5-fold and about 80-fold, by between about 10-fold and about 60-fold, by between about 10-fold and about 20-fold or by between about 20-fold and about 40-fold compared to the rate of a wild-type form of the recombinant N-OST. In some embodiments, the recombinant N-OST can increase the *in vivo* or *in vitro* rate of glycosylation of a carrier protein with a polysaccharide by more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold compared to the rate of a wild-type form of the recombinant N-OST.

[0126] In some embodiments, the rates of glycosylation of the recombinant N-OST and the wild-type form of the recombinant N-OST can be compared by comparing the recombinant N-OST's and the wild-type N-OST's rates of glycosylation of a carrier protein with a polysaccharide or oligosaccharide lacking an N-acetyl sugar at the reducing end.

[0127] In some embodiments, the recombinant N-OST's rate of glycosylation of a carrier protein with a polysaccharide or oligosaccharide lacking an N-acetyl sugar at the reducing end is compared to a wild-type N-OST's rate of glycosylation of a carrier protein with a polysaccharide or oligosaccharide having an N-acetyl sugar at the reducing end.

[0128] In some embodiments, the wild-type N-OST's rate of glycosylation of a carrier protein with a polysaccharide or oligosaccharide having an N-acetyl sugar at the reducing end is defined as a relative rate of 100%.

[0129] In some embodiments, the recombinant N-OST's rate of glycosylation of a carrier protein with a polysaccharide or oligosaccharide lacking an N-acetyl sugar at the reducing end is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the relative rate of a wild-type N-OST.

[0130] In some embodiments, the recombinant N-OST can increase the *in vivo* or *in vitro* yield of glycosylation of a carrier protein with a polysaccharide by between about 2-fold and about 100-fold, by between about 5-fold and about 80-fold, by between about 10-fold and about 60-fold, by between about 10-fold and about 20-fold or by between about 20-fold and about 40-fold compared to the yield achieved with a wild-type form of the recombinant N-OST. In some embodiments, the recombinant N-OST can increase the *in vivo* or *in vitro* yield of glycosylation of a carrier protein with a polysaccharide by more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold compared to the yield achieved with a wild-type form of the recombinant N-OST.

[0131] In some embodiments, the recombinant N-OST can yield an *in vivo* glycosylation level or an *in vitro* glycosylation level of the carrier protein of between about 1% to about 70%, of between about 3% to about 65%, of between about 5% to about 60%, of between about 5% to about 55%, of between about 10% to about 50%, of between about 15% to about 45%, of between about 20% to about 40%, or of between about 25% to about 35%. In some embodiments, the recombinant N-OST can yield an *in vivo* glycosylation level or an *in vitro* glycosylation level of the carrier protein of at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70%.

[0132] In some embodiments, the carrier protein comprises two or more N-glycosylation consensus sequences. In some embodiments, the recombinant N-OST can *in vitro* or *in vivo* glycosylate all N-glycosylation consensus sequences in the carrier protein. In some embodiments, the recombinant N-OST can glycosylate at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of all N-glycosylation consensus sequences in the carrier protein. In some embodiments, the recombinant N-OST can *in vitro* or *in vivo* glycosylate between about 10% and about 70%, between 20% and about 60%, or between about 30% and about 50% of all N-glycosylation consensus sequences in a carrier protein.

[0133] In some embodiments, the carrier protein comprises one or more N-glycosylation consensus sequences. In some embodiments, the carrier protein is a population of carrier proteins. In some embodiments, the recombinant N-OST can *in vitro* or *in vivo* glycosylate at least at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of all N-glycosylation consensus sequences in the carrier proteins of a population of carrier proteins. In some embodiments, the recombinant N-OST can *in vitro* or *in vivo* glycosylate between about 10% and about 70%, between 20% and about 60%, or between about 30% and about 50% of all N-glycosylation consensus sequences in the carrier proteins of a population of carrier proteins.

[0134] The recombinant N-OST can be from any organism having an N-OST. In some embodiments, the recombinant N-OST is from a eukaryotic organism. In some embodiments, the recombinant N-OST is from a prokaryotic organism. In some embodiments, the recombinant N-OST is from *Campylobacter jejuni (C. jejuni), Campylobacter coli (C. coli),* *Campylobacter lari (C. lari), Campylobacter upsaliensis (C. upsaliensis), Campylobacter curvus (C. curvus), Campylobacter concisus (C. concisus), Campylobacter hominis (C. hominis), Campylobacter gracilis (C. gracilis), Campylobacter showae (C.showae), Sulfurimonas autotrophica (S. autotrophica), Sulfurimonas denitrificans (S. denitrificans), Sulfurospirillum deleyianum (S. deleyianum), Sulfuricurvum kujiense (S. kujiense), Nautilia profundicola (N. profundicola), Sulfuvorum sp.* NBC37-1, *Wolinella succinogenes (W. succinogenes), Caminibacter mediatlanticus (C. mediatlanticus), Nitratiruptor sp.* SB155-2, *Helicobacter pullorum (H.pullorum), Helicobacter Canadensis (H. Canadensis), Helicobacter winghamensis (Helicobacter winghamensis), Desulfurobacterium thermolithotr (D. thermolithotr), Desulfomicrobium baculatum (D. baculatum), Desulfovibrio vulgaris (D. vulgaris), Desulfovibrio alkaliphilus (D. alkaliphilus), Desulfohalobium retbaense (D. retbaense), Deferribacter desulfuricans (D. desulfuricans), Desulfovibrio salexigenes (D. salexigenes), Desulfovibrio piger (D. salexigenes), Desulfovibrio aespoeensis (D. aespoeensis), Cand. Puniceispirillum marinum, Calditerrivibrio nitroreducens (C. nitroreducens)* or *Methanothermus fervidus (M. fervidus).*

[0135] In some embodiments, the recombinant N-OST is derived from a prokaryotic organism from the genus *Campylobacter.* In some embodiments, the recombinant N-OST is from *Campylobacter jejuni* or *Campylobacter lari (e.g.,* the *pglB* gene product PglB from *C. jejuni,* PglB_{Cj}, or from *C. lari,* PglB_{Cl}).

[0136] In some embodiments, the recombinant N-OST is a recombinant PglB_{Cj}, a recombinant PglB_{Cj} homologue or a recombinant version of a naturally occurring PglB_{Cj} variant. PglB_{Cj} homologues can comprise naturally occurring PglB_{Cj} homologues, *e.g.*, as exemplified in FIGs. 4 and 6, and non-naturally occurring PglB_{Cj} homologues. PglB_{Cj} homologues can comprise proteins having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to a PglB_{Cj} of SEQ ID NO:1.

[0137] In some embodiments, the recombinant N-OST is a recombinant PglB_{Cl}, a recombinant PglB_{Cl} homologue or a recombinant version of a naturally occurring PglB_{Cl} variant. PglB_{Cl} homologues can comprise naturally occurring PglB_{Cl} homologues, *e.g.*, as exemplified in FIG. 4, and non-naturally occurring PglB_{Cl} homologues. PglB_{Cl} homologues can comprise proteins having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to a PglB_{Cl} of SEQ ID NO:2.

[0138] Some amino acid positions are conserved in different members of a phylogenetic N-OST family. *See, e.g.,* FIG. 4. Some amino acid positions are conserved in at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or in 100% of members of a phylogenetic N-OST family. In some embodiments, amino acids in conserved amino acid positions are modified in the recombinant N-OSTs provided herein.

[0139] In some embodiments, the recombinant modified N-OST comprises a PglB fragment, *e.g.,* a PglB_{Cj} fragment or a PglB_{Cl} fragment. In some embodiments, the PglB fragment comprises at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, or at least 650 consequtive amino acids of a full-length PglB.

1. (a) PglB_{Cj} Modifications

[0140] In some embodiments, the modified N-OSTs described herein are modified wild-type N-OSTs, *e.g.*, wild-type PglB_{Cj}. In some embodiments, the wild-type PglB_{Cj} is a wild-type PglB_{Cj} of SEQ ID NO:1, or of a naturally occurring variant thereof:

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MLKKEYLKNP YLVLFAMIIL AYVFSVFCRF YWVWWASEFN EYFFNNQLMI ISNDGYAFAE
GARDMIAGFH QPNDLSYYGS SLSALTYWLY KITPFSFESI ILYMSTFLSS LVVIPTILLA
NEYKRPLMGF VAALLASIAN SYYNRTMSGY YDTDMLVIVL PMFILFFMVR MILKKDFFSL
IALPLFIGIY LWWYPSSYTL NVALIGLFLI YTLIFHRKEK IFYIAVILSS LTLSNIAWFY
QSAIIVILFA LFALEQKRLN FMIIGILGSA TLIFLILSGG VDPILYQLKF YIFRSDESAN
LTQGFMYFNV NQTIQEVENV DLSEFMRRIS GSEIVFLFSL FGFVWLLRKH KSMIMALPIL
VLGFLALKGG LRFTIYSVPV MALGFGFLLS EFKAIMVKKY SQLTSNVCIV FATILTLAPV
FIHIYNYKAP TVFSQNEASL LNQLKNIANR EDYVVTWWDY GYPVRYYSDV KTLVDGGKHL
GKDNFFPSFA LSKDEQAAAN MARLSVEYTE KSFYAPQNDI LKTDILQAMM KDYNQSNVDL
FLASLSKPDF KIDTPKTRDI YLYMPARMSL IFSTVASFSF INLDTGVLDK PFTFSTAYPL
DVKNGEIYLS NGVVLSDDFR SFKIGDNVVS VNSIVEINSI KQGEYKITPI DDKAQFYIFY

LKDSAIPYAQ FILMDKTMFN SAYVQMFFLG NYDKNLFDLV INSRDAKVFK LKIYPYDVPD
YA
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[0141] In some embodiments, one or more of amino acids Y77, S80, S196, N311, Y462, H479, K522, G476 or G477 of PglB_{Cj}, or any combination thereof, are modified.

[0142] In some embodiments, the amino acid N311 of PglB_{Cj} is modified. In some embodiments, the modification of N311 is a N311V or a N311I substitution. In some embodiments, the modification of N311 is a N311V substitution.

[0143] In some embodiments, the amino acids N311 and Y77 of PglB_{Cj} are modified. In some embodiments, the modification of Y77 is a Y77H, Y77T, Y77W, Y77R, Y77K, Y77A, or Y77G substitution. In some embodiments, the modification of Y77 is a Y77H substitution.

[0144] In some embodiments, the amino acids N311 and S80 of PglB_{Cj} are modified. In some embodiments, the modification of S80 is a S80R substitution or a S80H substitution. In some embodiments, the modification of S80 is a S80R substitution.

[0145] In some embodiments, the recombinant PglB_{Cj} comprises a modification in at least one amino acid of the Q287LKFYxxR294 motif of $PglB_{Cj}$. In some embodiments, at least one amino acid of Q287, L288 or K289 of $PglB_{Cj}$ are modified. In some embodiments, the recombinant $PglB_{Cj}$ comprises a Q287P, Q287K, Q287R, L288M, L288F, L288I, L288C, K289R, K289N, K289Q or R294K subsitution.

[0146] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution.

[0147] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution and a Y77H substitution.

[0148] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution and a S80R substitution.

[0149] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising a Y77H substitution and a Q287P substitution.

[0150] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising a S80R substitution and a Q287P substitution.

[0151] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution, a S80R substitution and a Q287P substitution.

[0152] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution, a Y77H substitution and a Q287P substitution.

[0153] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V mutation, aY77H substitution, a S80R substitution and a Q287P substitution.

[0154] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising an N311V substitution, a Y77H substitution, a S80R substitution, a Q287P substitution and a K289R substitution.

[0155] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising g a N311V substitution and a A699V substitution.

[0156] In another embodiment, provided herein is a recombinant $PglB_{Cj}$ comprising a K482R substitution and a D483H substitution.

[0157] In some embodiments, the substitution can be a conservative amino acid substitution *(e.g.,* from one basic amino acid to another basic amino acid). In some embodiments, the substitution can be a non-conservative amino acid substitution (*e.g.*, from a basic amino acid to an acidic amino acid).

(b) PglB$_{Cl}$ Modifications

[0158] In some embodiments, the modified N-OSTs described herein are modified wild-type NOSTs, *e.g.,* wild-type PglB$_{Cl}$ (PglB of *Campylobacter lari).* In some embodiments, the wild-type PglB$_{Cj}$ is a wild-type PglB$_{Cl}$ of SEQ ID NO:2, or of a naturally occurring variant thereof:

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MKLQQNFTDN NSIKYTCILI LIAFAFSVLC RLYWVAWASE FYEFFFNDQL
MITTNDGYAF AEGARDMIAG FHQPNDLSYF GSSLSTLTYW LYSILPFSFE
SIILYMSAFF ASLIVVPIIL IAREYKLTTY GFIAALLGSI ANSYYNRTMS
GYYDTDMLVL VLPMLILLTF IRLTINKDIF TLLLSPVFIM IYLWWYPSSY
SLNFAMIGLF GLYTLVFHRK EKIFYLTIAL MIIALSMLAW QYKLALIVLL
FAIFAFKEEK INFYMIWALI FISILILHLS GGLDPVLYQL KFYVFKASDV
QNLKDAAFMY FNVNETIMEV NTIDPEVFMQ RISSSVLVFI LSFIGFILLC
KDHKSMLLAL PMLALGFMAL RAGLRFTIYA VPVMALGFGY FLYAFFNFLE
KKQIKLSLRN KNILLILIAF FSISPALMHI YYYKSSTVFT SYEASILNDL
KNKAQREDYV VAWWDYGYPI RYYSDVKTLI DGGKHLGKDN FFSSFVLSKE
QIPAANMARL SVEYTEKSFK ENYPDVLKAM VKDYNKTSAK DFLESLNDKD
FKFDTNKTRD VYIYMPYRML RIMPVVAQFA NTNPDNGEQE KSLFFSQANA

IAQDKTTGSV MLDNGVEIIN DFRALKVEGA SIPLKAFVDI ESITNGKFYY
NEIDSKAQIY LLFLREYKSF VILDESLYNS SYIQMFLLNQ YDQDLFEQIT
NDTRAKIYRL KR
```

[0159] In some embodiments, one or more of amino acids Y79, S82, N314, K488, or D489 of PglB$_{Cj}$, or any combination thereof, are modified.

[0160] In some embodiments, the amino acid N314 of PglB$_{Cl}$ is modified. In some embodiments, the modification of N314 is a N314V or a N314I substitution. In some embodiments, the modification of N314 is a N314V substitution.

[0161] In some embodiments, the amino acids N314 and Y797 of PglB$_{Cl}$ are modified. In some embodiments, the modification of Y79 is a Y79H, Y79T, Y79W, Y79R, Y79K, Y79A, or Y79G substitution. In some embodiments, the modification of Y79 is a Y79H substitution.

[0162] In some embodiments, the amino acids N314 and S82 of PglB$_{Cl}$ are modified. In some embodiments, the modification of S82 is a S82R substitution or a S82H substitution. In some embodiments, the modification of S82 is a S82R substitution.

[0163] In some embodiments, the recombinant PglB$_{Cl}$ comprises a modification in at least one amino acid of the QLKFYxxR motif of PglB$_{Cj}$. In some embodiments, at least one the amino acid Q289 is modified. In some embodiments, the recombinant PglB$_{Cl}$ comprises a Q289P, Q289K, or Q289R subsitution.

[0164] In another embodiment, provided herein is a recombinant PglB$_{Cl}$ comprising an N314V substitution.

[0165] In another embodiment, provided herein is a recombinant PglB$_{Cl}$ comprising an N314V substitution and a Y79H substitution.

[0166] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising an N314V substitution and a S82R substitution.

[0167] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising a Y79H substitution and a Q289P substitution.

[0168] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising a S82R substitution and a Q289P substitution.

[0169] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising an N314V substitution, a S82R substitution and a Q289P substitution.

[0170] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising an N314V substitution, a Y79H substitution and a Q289P substitution.

[0171] In another embodiment, provided herein is a recombinant PglB$_{Cj}$ comprising an N314V mutation, aY79H substitution, a S82R substitution and a Q289P substitution.

[0172] In some embodiments, the substitution can be a conservative amino acid substitution *(e.g.,* from one basic amino acid to another basic amino acid). In some embodiments, the substitution can be a non-conservative amino acid substitution (*e.g.*, from a basic amino acid to an acidic amino acid).

6.2 Methods of Screening

[0173] In another aspect, provided herein is a method of screening a library of recombinant N-OSTs provided herein, including contacting each member of the library of recombinant NOSTs with a carrier protein and an oligosaccharide or a polysaccharide lacking an N-acetyl sugar at its reducing end to produce a bioconjugate.

[0174] In some embodiments, the bioconjugate is a N-glycosylated carrier protein.

[0175] The oligosaccharides and polysaccharides can include any oligosaccharide or polysaccharide described herein. *See, e.g.,* Section 6.4.

[0176] The carrier proteins can comprise any carrier protein described herein. *See, e.g.,* Section 6.5.

[0177] In some embodiments, the contacting occurs *in vitro.* In some embodiments, the contacting occurs *in vivo.* In some embodiments, the contacting occurs in a host cell described herein. In some embodiments, the host cell is a prokaryotic cell. In some embodiments, the host cell is an *E. coli* cell.

[0178] In some embodimens, the library of recombinant N-OSTs comprises one or more recombinant N-OSTs provided herein. In some embodiments, the library of recombinant N-OSTs comprises at least 2, at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 500, at least 750 or at least 1,000 recombinant N-OSTs. In some embodiments, the library of recombinant N-OSTs comprises between about 2 and about 1,000, between about 10 and about 800, between about 50 and about 600, between about 100 and about 400, or between about 100 and about200 recombinant N-OSTs.

[0179] In some embodiments, the method further comprises determining the rate or yield of production of the bioconjugate. In some embodiments, the method further comprises determining the its level of conjugation (*e.g.*, glycosylation level in percent glycosylated carrier protein) of the bioconjugate. Methods for determining the rate or yield of production of a bioconjugate or the level of bioconjugate conjugation are known in the art. *See, e.g.,* Section 6.9, Section 6.10, Section 6.12, and Examples 2-3.

[0180] In some embodiments, the method further comprises selecting one or more recombinant N-OSTs form the library of recombinant N-OSTs.

[0181] In some embodiments, the one or more recombinant N-OSTs are selected if the recombinant N-OST yields bioconjugate at a rate that is between about 2-fold and about 100-fold, between about 5-fold and about 80-fold, between about 10-fold and about 60-fold, between about 10-fold and about 40-fold, between about 10-fold and about 30-fold, or between about 10-fold and about 20-fold faster than the rate of a wild-type N-OST. In some embodiments, the one or more recombinant N-OSTs are selected if the recombinant N-OST yields bioconjugate at a rate that is more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold faster than the rate of a wild-type N-OST.

[0182] In some embodiments, the one or more recombinant N-OSTs are selected if the N-OST mutant yields between about 2-fold and about 100-fold, between about 5-fold and about 80-fold, between about 10-fold and about 60-fold, between about 10-fold and about 40-fold, between about 10-fold and about 30-fold, or between about 10-fold and about 20-fold the amount of bioconjugate compared to a wild-type N-OST. In some embodiments, the one or more N-oligosaccharyl transferase mutants are selected if the N-OST mutant yields more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-

fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold the amount of bioconjugate compared to a wild-type N-OST.

[0183] In some embodiments, the one or more recombinant N-OST is selected if the recombinant N-OST glycosylates between about 1% and about 70%, between about 3% and about 65%, between about 5% and about 60%, between about 10% and about 55%, between about 15% and about 50%, between about 20% and about 45%, between about 20% and about 40%, or between about 25% and about 35% of carrier protein *in vitro (e.g.,* in a reaction vessel) or *in vivo (e.g.,* in a host cell). In some embodiments, the one or more recombinant N-OST is selected if the recombinant N-OST glycosylates at least 1%, at least 3%, 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of carrier protein *in vitro (e.g.,* in a reaction vessel) or *in vivo (e.g.,* in a host cell).

[0184] In another aspect, provided herein is a method of identifying a recombinant N-OST provided herein that has a modified substrate selectivity relative to a wild-type form of the recombinant N-OST, including substituting one or more amino acids (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more amino acids) whose side chains are located within a 2.5-4.0 Å distance from one of the three terminal monosaccharide units at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein in a structural model of a complex of the recombinant N-OST and the N-glycosylated carrier protein.

[0185] In some embodiments, the N-OST mutant has a modified substrate selectivity *in vitro.* In some embodiments, the N-OST mutant has a modified substrate selectivity *in vivo.*

6.3 Structural models

[0186] The structural models used to describe the recombinant N-OSTs disclosed herein comprise a complex of the recombinant N-OST and a bound N-glycosylated carrier protein and can be obtained using any methods known to a skilled artisan. For example, the structural model can be obtained using X-ray crystallography or nuclear magnetic resonance spectroscopy (NMR). Exemplary methods for obtaining structural models of protein complexes are described, *e.g.,* in Bernhard Rupp, Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology, Garland Science, 1 edition (October 20, 2009); Eaton E. Lattman and Patrick J. Loll; Protein Crystallography: A Concise Guide, Johns Hopkins University Press; 1 edition (March 26, 2008); Arthur G. Palmer III and Wayne J. Fairbrother; Protein NMR Spectroscopy, Second Edition: Principles and Practice; Academic Press, 2 edition (December 28, 2005). The structural model can, *e.g.,* be an x-ray structure model or an NMR structur model of a complex of an N-OST and a bound N-glycosylated carrier. In some embodiments, the structural model can be a homology model of a complex of the recombinant

N-OST and the bound N-glycosylated carrier protein. *See, e.g.,* Example 1 and FIG. 2. The oligosaccharide or polysaccharide and carrier protein components of an N-glycosylated carrier protein can be modeled in a product conformation or in a substrate conformation.

[0187] The structural models used to describe the recombinant N-OSTs disclosed herein can comprise any N-OST, any carrier protein, or any oligosaccharide or polysaccharide disclosed herein. *See, e.g.,* Sections 6.1, 6.4, and 6.5.

[0188] The structural model can comprise the model of a full-length recombinant N-OST or of a fragment thereof. The structural model can be built, *e.g.,* using a recombinant N-OST, a recombinant wild-type N-OST, or a N-OST purified from an N-OST expressing organism. In some embodiments, the structural model comprises the catalytic site of the N-OST. The structural model can model a N-OST from any organism having an N-OST. The structural model can model any recombinant N-OST described herein. *See, e.g.,* FIG. 4. In some embodiments, the structural model is a homology model generated using the experimentally solved structure of *C. lari* PglB (PDBid 3RCE) as template. *See, e.g.,* Example 1. The structural model can be built using the oligosaccharide or polysaccharides and/or the carrier proteins described herein. The N-OST, carrier protein and oligosaccharide or polysaccharide component used to build the structural model can all be from the same organism or from two or three different organisms.

[0189] In some embodiments, the bound N-glycosylated carrier protein comprises a natural oligosaccharide or polysaccharide component (an oligosaccharide or polysaccharide component from the same organism as the N-OST). In some embodiments, the bound N-glycosylated carrier protein comprises a heterologous oligosaccharide or polysaccharide component (an ogosaccharide or polysaccharide component from a different organism than the N-OST). In some embodiments, the bound N-glycosylated carrier protein comprises a natural oligosaccharide or polysaccharide component of *Campylobacter jejuni (C. jejuni;* an oligosaccharide or polysaccharide component from *C. jejuni).* In some embodiments, the bound N-glycosylated carrier protein comprises a heterologous oligosaccharide or polysaccharide component of *C. jejuni* (an oligosaccharide or polysaccharide component that is not from *C. jejuni).*

[0190] In the structural model, physical distances, *e.g.*, between certain N-OST amino acid side chains and the monosaccharide unit at the reducing end of the oligosaccharide or polysaccharide component of a bound N-glycosylated carrier protein can be determined using any method or software tools known to a skilled artisan. *See, e.g.,* Chang, G. et al., An internal coordinate Monte-Carlo Method for Searching Conformational Space. J. Am. Chem. Soc, 1989, 111, 4379; Saunders, M., et al., Conformations of cycloheptadecane: A Comparison of Methods for Conformational Searching. J. Am. Chem. Soc. 1990, 112, 1419.

6.4 Oligosaccharides and Polysaccharides

[0191] The oligosaccharides that can be linked to a carrier protein by the recombinant N-OSTs provided herein can have between 2 and 100 monosaccharide units, *e.g.,* 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 or 100 monosaccharide units. The polysaccharides that can be linked to a carrier protein by the recombinant N-OSTs provided provided herein can have more than 100 monosaccharide units, *e.g.,* 101, 110, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 monosaccharide units or more.

[0192] The carrier proteins or N-OSTs can comprise any N-OST or any carrier protein disclosed herein. *See, e.g.,* Section 6.1 and 6.5.

[0193] In some embodiments, the sugar at the reducing end of the oligosaccharide or polysaccharide is a pentose, hexose, or heptose. In some embodiments, the sugar at the reducing end of the oligosaccharide or polysaccharide is an aldopentose or a ketopentose. In some embodiments, the pentose is a D-arabinose, a D-lyxose, a D-ribose, a D-xylose, a D-ribulose, or a D-Xylulose. In some embodiments, the sugar at the reducing end of the oligosaccharide or polysaccharide is an aldohexose or a ketohexose. In some embodiments, the hexose is, *e.g.,* a D-allose, D-altrose, D-glucose, D-mannose, D-gulose, D-idose, D-galactose, D-talose, D-psicose, D-fructose, D-sorbose or D-tagatose. In some embodiments, the sugar at the reducing end of the oligosaccharide or polysaccharide is a deoxy or a di-deoxy sugar, such as, *e.g.,* a rhamnose, a fucose, or an abequose. In some embodiments, the sugar at the reducing end of the oligosaccharide or polysaccharide is an aldoheptose or a ketoheptose. In some embodiments, the heptose is a mannoheptulose.

[0194] The oligosaccharides and polysaccharides that can be linked to a carrier protein by the recombinant N-OSTs provided herein can be from any organism, *e.g.*, a prokaryotic organism or a eukaryotic organism. In some embodiments, the oligosaccharide or polysaccharide is from a pathogenic organism, *e.g.*, a human pathogen or an animal pathogen *(e.g.,* a farm animal or a pet). In some embodiments, the oligosaccharide or polysaccharide is from a bacterial organism. In some embodiments, the oligosaccharide or polysaccharide can be from *E. coli, Salmonella* sp (*e.g., S. enterica* subsp. *Enterica, S.* enterica subsp. *Salamae, S. enterica* subsp. arizonae, *S. enterica* subsp. Diarizonae, *S. enterica* subsp. Houtenae, *S. bongori,* and *S. enterica* subsp. Indica, Pseudomonas sp (*P. aeruginosa*), *Klebsiella* sp. (*e.g., K. pneumonia*), *Acinetobacter, Chlamydia trachomatis, Vibrio cholera, Listeria* sp., *e.g., L. monocytogenes, Legionella pneumophila, Bordetella parapertussis, Burkholderia mallei* and *pseudomallei, Francisella tularensis, Campylobacter* sp. (*C. jejuni); Clostridium difficile, Staphylococcus aureus, Streptococcus pyrogenes, E. coli, Streptococcus agalacticae, Neisseria meningitidis, Candida albicans, Haemophilus influenza, Enterococcus faecalis, Borrelia burgdorferi, Neisseria meningitidis, Haemophilus influenza, Leishmania major.*

[0195] In some embodiments, the oligosaccharide or polysaccharide comprises an antigen, *e.g.,* an epitope that is immunogenic in a human or an animal *(e.g.,* a farm animal or a pet). In some embodiments, the oligosaccharide or the polysaccharide comprises an O antigen of *E. coli* (*e.g.,* O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, 012, 013, 014, 015, 016, 017, 018, O19, O20, O21, O22, O23, O24, O25, O26, O27, O28, O29, O30, O32, O33, O34, O35, O36,

O37, O38, O39, O40, O41, O42, O43, O44, O45, O46, O48, O49, O50, O51, O52, O53, O54, O55, O56, O57, O58, O59, O60, O61, O62, O63, O64, O65, O66, O68, O69, O70, O71, O73, O74, O75, O76, O77, O78, O79, O80, O81, O82, O83, O84, O85, O86, O87, O88, O89, O90, O91, O92, O93, O95, O96, O97, O98, O99, O100, O101, 0102, 0103, 0104, 0105, 0106, O107, O108, O109, O110, O111, O112, O113, O114, O115, O116, O117, O118, O119, O120, O121, O123, O124, O125, O126, O127, O128, O129, O130, O131, O132, O133, O134, O135, O136, O137, O138, O139, O140, O141, O142, O143, O144,O145, O146, O147, O148, O149, O150, O151, O152, O153, O154, O155, O156, O157, O158, O159, O160, O161, O162, O163, O164, O165, O166, O167, O168, O169, O170, O171, O172, O173, O174, O175, O176, O177, O178, O179, O180, O181, O182, O183, O184, O185, O186, O187), Salmonella sp *(S. enterica* subsp. Enterica, *S. enterica* subsp. Salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae, *S. bongori,* or *S. enterica* subsp. indica antigens and O types 1-67, as detailed in [44], *Pseudomonas* sp. (*P. aeruginosa* O serotypes 1-20 [45]), *Klebsiella* sp. (*e.g., K. pneumonia* serotypes O1, O2 (and subserotypes), O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, [46]), Acinetobacter O antigens (*e.g., A. baumannii* O antigens identified in [47]), *Chlamydia trachomatis* O antigens (serotypes A, B, C, D, E, F, G, H, I J, K, LI, L2, L3), *Vibrio cholera* O antigens O1 to 155, *Listeria* sp., in particular *L. monocytogenes* type 1, 2, 3, 4 and subserotypes thereof, *Legionella pneumophila* serotypes 1 to 15 O antigens, *Bordetella parapertussis* O antigens, *Burkholderia mallei* and *pseudomallei* O antigens, *Francisella tularensis, Campylobacter* sp. (*C. jejuni*); Capsular polysaccharides of *Clostridium difficile* (serotypes A, G, H, K, S1, S4, D, Cd-5, K Toma *et al.* 1988, and *C. perfringens* serotypes A, B, C, D und E), *Staphylococcus aureus* type 5 and 8, *Streptococcus pyrogenes* (group B streptococcus capsular serotype polysaccharides), *E. coli, Streptococcus agalacticae* (group A streptococcal capsular polysaccharides), *Neisseria meningitidis* (serotypes A, B, C, W, Y, X), *Candida albicans, Haemophilus influenza, Enterococcus faecalis* capsular polysaccharides type I-V; and other surface polysaccharide structures, *e.g.,* the *Borrelia burgdorferi* glycolipids ([48]), *Neisseria meningitidis* pilin O glycan [49, 50] and lipooligosaccharide (LOS), *Haemophilus influenza* LOS, *Leishmania major* lipophosphoglycan [51, 52]), tumor associated carbohydrate antigens (malaria glycosyl phosphatidylinositol, mycobacterium tuberculosis arabinomannan [53].

[0196] In some embodiments, the oligosaccharide or polysaccharide is a *Staphylococcus aureus (S. aureus)* or a *Salmonella enterica sv. (S. enterica sv.)* polysaccharide. In some embodiments, the polysaccharide is a *S. aureus* CP5 or a *S. enterica sv.* Typhimurium LT2 polysaccharide.

[0197] In some embodiments, the oligosaccharide or polysaccharide comprises an N-acetyl sugar at the reducing end. In some embodiments, the oligosaccharide or polysaccharide comprising the N-acetyl sugar at the reducing end can comprise, *e.g.,* an O antigen *of E. coli (e.g.,* O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, O13, O14, O15, O16, O17, O18, O19, O20, O21, O22, O23, O24, O25, O26, O27, O28, O29, O30, O32, O33, O34, O35, O36, O37, O38, O39, O40, O41, O42, O43, O44, O45, O46, O48, O49, O50, O51, O52, O53, O54, O55, O56, O57, O58, O59, O60, O61, O62, O63, O64, O65, O66, O68, O69, O70, O71, O73, O74, O75, O76, O77, O78, O79, O80, O81, O82, O83, O84, O85, O86, O87, O88, O89, O90,

O91, O92, O93, O95, O96, O97, O98, O99, O100, O101, O102, O103, O104, O105, O106, O107, O108, O109, O110, O111, O112, O113, O114, O115, O116, O117, O118, O119, O120, O121,O123, O124, O125, O126, O127, O128, O129, O130, O131, O132, O133, O134, O135, O136, O137, O138, O139, O140, O141, O142, O143, O144,O145, O146, O147, O148, O149, O150, O151, O152, O153, O154, O155, O156, O157, O158, O159, O160, O161, O162, O163, O164, O165, O166, O167, O168, O169, O170, O171, O172, O173, O174, O175, O176, O177, O178, O179, O180, O181, O182, O183, O184, O185, O186, O187), a capsular polysaccharide of *Staphylococcus aureus (S. aureus) (e.g.,* CP5 or CP8), a capsular polysaccharide of *Francisella tularensis Schu4,* a capsular polysaccharide of *S. pneumoniae capsules (e.g.,* CP1, 4, 5, 12,25, 38, 44, 45 or 46), a *Neisseria meningitidis* pilin O glycan [49, 50], a *Burkholderia mallei* and *pseudomallei* O antigen, a *Bordetella parapertussis* O antigen, a *Legionella pneumophila* serotypes 1 to 15 O antigen, a *Listeria* sp. O antigen, in particular an O antigen of *L. monocytogenes* type 1, 2, 3, 4, an O antigen of *Pseudomonas* sp. (*P. aeruginosa* O serotypes 1-20 [45]), an O antigen of *Klebsiella* sp. (*e.g., K. pneumonia* serotypes O1, O2 (and subserotypes), O3, O4, O5, O6, O7, O8, O9, O10, O11, 012, [46]), an O antigen of Shigella sp. (*e.g., S. dysenteriae, S. sonnei, S. flexneri, S. boydii*), an Acinetobacter O antigen (*e.g., A. baumannii* O antigens identified in [47]), or an O antigen of *Listeria* sp.

[0198] N-acetyl sugars can comprise an amino-acetyl (N-acetyl) substituent at one or more carbon atoms of the sugar. For example, an N-acetyl sugar can comprise an N-acetyl substituent at the C2-atom of a monosaccharide unit, such as a glucose unit (N-acetylglucosamine).

[0199] In some embodiments, the oligosaccharide or polysaccharide comprises a sugar at the reducing end that is not N-acetylated. In some embodiments, the oligosaccharide or polysaccharide comprising the non-N-acetylated sugar at the reducing end can comprise, *e.g., E. coli* O20, an antigen of Salmonella sp (*e.g., S. enterica* subsp. Enterica, *S. enterica* subsp. Salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae, *S. bongori,* or *S. enterica* subsp. Indica or *S.* Typhi), an O antigen of type 1-67, a capsular polysaccharide of *group A Streptococcus (S. pyrogenes), group B Streptococcus,* and of *S. pneumoniae CPS serotypes (encoding wchA, wcjG, or wcjH in their capsular gene clusters, i.e. all serotypes except CP1, 4, 5, 12, 25, 38, 44, 45, 46),* or a *Salmonella enterica sv. (S. enterica sv.*) O antigen.

[0200] In some embodiments, the oligosaccharide or polysaccharide comprises a *S. aureus* CP5 or a *S. enterica sv.* Typhimurium LT2 polysaccharide, a *Vibrio cholera* O antigen *(e.g.,* O1 to 155), or a *Listeria* sp. O antigen *(e.g., L. monocytogenes* type 1, 2, 3, 4).

[0201] In some embodiments, the oligosaccharide or polysaccharide comprises a D-N-acetylfucosamine (D-FucNAc) residue at its reducing end, such as, *e.g.*, capsular polysaccharides of *S. aureus* serotypes 5, 8 or *P. aeruginosa* O antigen serotypes O2, O5, O11, 016.

[0202] In some embodiments, the oligosaccharide or polysaccharide comprises a 4-amino-d-

N-acetylfucosamine (D-FucNAc4N) residue at its reducing end, such as, *e.g.*, certain oligosaccharides or polysaccharides from *S. pneumoniae,* like serotype 1, *Shigella sonnei* O antigen, or *Plesiomonas shigelloides* 017.

[0203] In some embodiments, the oligosaccharide or polysaccharide comprises a D-N-acetylquinosamine (D-QuiNAc) residue at its reducing end, such as, *e.g.,* like *P. aeruginosa* O antigen serotypes O6, O1, or *Francisella tularensis* serotype Schu4.

[0204] In some embodiments, the oligosaccharide or polysaccharide comprises a galactose residue at its reducing end, such as, *e.g., S. enterica* LT2.

[0205] In some embodiments, the oligosaccharide or polysaccharide comprises a *S. pneumoniae* capsular polysaccharide serotype 5, *E. coli* O1, O2, Cronobacter sakazakii O5, *i.e.,* poly- and oligosaccharide with a reducing end D-GlcNAc linked to 1-4 to a L-Rhamnose in beta configuration.

6.5 Carrier Proteins

[0206] Carrier proteins can be linked to oligosaccharides or polysaccharides by the recombinant N-OSTs provided herein. *See, e.g.,* Section 6.1.

[0207] The carrier protein can be any natural carrier protein (from the same organism as the N-OST) or any heterologous carrier protein (from a different organism than the N-OST). In some embodiments, the carrier protein is an immunogen. Carrier proteins can be full-length proteins or fragments thereof. Exemplary carrier proteins comprise, without limitation, exotoxin A of *P. aeruginosa* (EPA), CRM197, diphtheria toxoid, tetanus toxoid, detoxified hemolysin A of *S. aureus,* clumping factor A, clumping factor B, *E. coli* FimH, *E. coli* FimHC, *E. coli* heat labile enterotoxin, detoxified variants *of E. coli* heat labile enterotoxin, Cholera toxin B subunit (CTB), cholera toxin, detoxified variants of cholera toxin, *E. coli* sat protein, the passenger domain *of E. coli* sat protein, *C. jejuni* AcrA, and *C. jejuni* natural glycoproteins. In some embodiments, the carrier protein is exotoxin A of *P. aeruginosa* (EPA).

[0208] In some embodiments, the carrier proteins N-glycosylated by a recombinant N-OST described herein are modified, *e.g.*, modified in such a way that the protein is less toxic and or more susceptible to glycosylation, *etc.* In some embodiments, the carrier proteins are modified such that the number of glycosylation sites in the carrier proteins is maximized in a manner that allows for lower concentrations of the protein to be administered, *e.g.*, in an immunogenic composition, in its bioconjugate form. Accordingly in certain embodiments, the carrier proteins described herein are modified to comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more glycosylation sites than would normally be associated with the carrier protein (*e.g.*, relative to the number of glycosylation sites associated with the carrier protein in its native/natural, *e.g.*, "wild-type" state). In some embodiments, introduction of glycosylation sites is accomplished by insertion of glycosylation consensus sequences (*e.g.*, (i) the consensus sequence Asn-X-Ser(Thr), wherein

X is are independently selected from any amino acid except Pro; or (ii) the consensus sequence D/E-X-N-Z-S/T, wherein X and Z are independently selected from any amino acid except Pro) anywhere in the primary structure of the protein. Introduction of such glycosylation sites can be accomplished by, *e.g.,* adding new amino acids to the primary structure of the protein (the glycosylation sites are added, in full or in part), or by modifying existing amino acids in the protein in order to generate the glycosylation sites (amino acids are not added to the protein, but selected amino acids of the protein are mutated so as to form glycosylation sites). Those of skill in the art will recognize that the amino acid sequence of a protein can be readily modified using approaches known in the art, *e.g.*, recombinant approaches that comprise modification of the nucleic acid sequence encoding the protein. In specific embodiments, glycosylation consensus sequences are introduced into specific regions of the carrier protein, *e.g.*, surface structures of the protein, at the N or C termini of the protein, and/or in loops that are stabilized by disulfide bridges at the base of the protein. In certain embodiments, the classical 5 amino acid glycosylation consensus sequence may be extended by lysine residues for more efficient glycosylation, and thus the inserted consensus sequence may encode 5, 6, or 7 amino acids that should be inserted or that replace acceptor protein amino acids.

[0209] The N-OSTs can comprise any N-OST disclosed herein. *See, e.g.,* Section 6.1.

[0210] In some embodiments, the carrier proteins comprise a "tag," a sequence of amino acids that allows for the isolation and/or identification of the carrier protein. For example, adding a tag to a carrier protein described herein can be useful in the purification of that protein and, hence, the purification of conjugate vaccines comprising the tagged carrier protein. Exemplary tags that can be used herein comprise, without limitation, histidine (HIS) tags (*e.g.,* hexa histidine-tag, or 6XHis-Tag), FLAG-TAG, and HA tags. In certain embodiments, the tags used herein are removable, *e.g.*, removal by chemical agents or by enzymatic means, once they are no longer needed, *e.g.,* after the protein has been purified.

6.6 Nucleic Acids

[0211] In another aspect, provided herein are nucleic acids encoding the recombinant N-OSTs provided herein (*e.g.*, Section 6.1).

[0212] In some embodiments, the nucleic acids encode a recombinant PglB$_{Cj}$ wherein one or more of amino acids Y77, S80, S196, N311, Y462, H479, K522, G476 or G477 are modified.

[0213] In some embodiments, the nucleic acids encode a PglB$_{Cj}$ wherein the amino acid N311 of PglB$_{Cj}$ is modified. In some embodiments, the modification of N311 is a N311V or a N311I substitution. In some embodiments, the modification of N311 is a N311V substitution.

[0214] In some embodiments, the nucleic acids encode a recombinant PglB$_{Cj}$ wherein the amino acids N311 and Y77 are modified. In some embodiments, the modification of Y77 is a Y77H, Y77T, Y77W, Y77R, Y77K, Y77A, or Y77G substitution. In some embodiments, the modification of Y77 is a Y77H substitution.

[0215] In some embodiments, the nucleic acids encode a recombinant PglB$_{Cj}$ wherein the amino acids N311 and S80 of PglB$_{Cj}$ are modified. In some embodiments, the modification of S80 is a S80R substitution or a S80H substitution. In some embodiments, the modification of S80 is a S80R substitution.

[0216] In some embodiments, the nucleic acids encode a recombinant PglB$_{Cj}$ wherein the recombinant PglB$_{Cj}$ comprises a modification in at least one amino acid of the Q287LKFYxxR294 motif of PglB$_{Cj}$. In some embodiments, the nucleic acids encode a recombinant PglB$_{Cj}$ wherein at least one amino acid of Q287, L288 or K289 of PglB$_{Cj}$ is modified. In some embodiments, the recombinant PglB$_{Cj}$ comprises a Q287P, Q287K, Q287R, L288M, L288F, L288I, L288C, K289R, K289N, K289Q or R294K subsitution.

[0217] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V substitution.

[0218] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V substitution and a Y77H substitution.

[0219] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V substitution and a S80R substitution.

[0220] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising a Y77H substitution and a Q287P substitution.

[0221] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising a S80R substitution and a Q287P substitution.

[0222] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V substitution, a S80R substitution and a Q287P substitution.

[0223] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V substitution, a Y77H substitution and a Q287P substitution.

[0224] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising an N311V mutation, aY77H substitution, a S80R substitution and a Q287P substitution.

[0225] In another embodiment, provided herein is a nucleic acid encoding a recombinant

PglB$_{Cj}$ comprising an N311V substitution, a Y77H substitution, a S80R substitution, a Q287P substitution and a K289R substitution.

[0226] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising a N311V substitution and a A699V substitution.

[0227] In another embodiment, provided herein is a nucleic acid encoding a recombinant PglB$_{Cj}$ comprising a K482R substitution and a D483H substitution.

6.7 Host Cells

[0228] In another aspect, provided herein is a host cell comprising a recombinant N-OST provided herein. In some embodiments, the host cell comprises two or more recombinant N-OSTs provided herein (*e.g.,* 2, 3, 4, 5, 6, 7, 8, 9, 10 or more recombinant N-OSTs).

[0229] In another aspect, provided herein is a host cell comprising a nucleic acid provided herein *(e.g.,* encoding a recombinant N-OST provided herein, *e.g.,* Section 6.1). *See, e.g.,* Section 6.6. In some embodiments, the host cell comprises two or more nucleic acids provided herein *(e.g.,* 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acids).

[0230] In some embodiments, the host cell comprises one or more further enzymes useful for bioconjugate production or carrier protein N-glycosylation (*e.g.*, a glycosyltransferase). In some embodiments, at least one of the further enzymes useful for bioconjugate production is a recombinant enzyme. In some embodiments, the host cell comprises two or more further enzymes useful for bioconjugate production (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more further enzymes).

[0231] In some embodiments, the host cell is a prokaryotic cell. In some embodiments, the host cell is an *E. coli* cell. In some embodiments, the host cell comprises a recombinant N-OST provided herein. *See, e.g.,* Section 6.1. In some embodiments, the host cell comprises a carrier protein and a recombinant N-OST provided herein. *See, e.g.,* Section 6.1 and 6.5. In some embodiments, the host cell comprises a carrier protein, a recombinant N-OST provided herein, and a recombinant glycosyltransferase. In some embodiments, the recombinant N-OST is a recombinant PglB$_{Cj}$. *See, e.g.,* Section 6.1(a).

[0232] In certain embodiments, the host cells used to produce the bioconjugates described herein are engineered to comprise heterologous nucleic acids, *e.g.*, heterologous nucleic acids that encode one or more carrier proteins and/or heterologous nucleic acids that encode one or more proteins, *e.g.*, genes encoding one or more proteins. In some embodiments, heterologous nucleic acids that encode proteins involved in glycosylation pathways (*e.g.*, prokaryotic and/or eukaryotic glycosylation pathways) are introduced into the host cells described herein. Such nucleic acids can encode proteins including, without limitation, oligosaccharyl transferases and/or glycosyltransferases. Heterologous nucleic acids (*e.g.*, nucleic acids that encode carrier proteins and/or nucleic acids that encode other proteins, *e.g.*, proteins involved in glycosylation) can be introduced into the host cells described herein using any methods known to those of skill in the art, *e.g.*, electroporation, chemical transformation by heat shock, natural transformation, phage transduction, and conjugation. In some embodiments, heterologous nucleic acids are introduced into the host cells described herein using a plasmid, *e.g.*, the heterologous nucleic acids are expressed in the host cells by a plasmid (*e.g.*, an expression vector). In some embodiments, heterologous nucleic acids are introduced into the host cells described herein using the method of insertion described in International Patent application Publication No. WO 2014/057109.

[0233] In certain embodiments, additional modifications can be introduced (*e.g.*, using recombinant techniques) into the host cells described herein. For example, host cell nucleic acids (*e.g.*, genes) that encode proteins that form part of a possibly competing or interfering glycosylation pathway (*e.g.*, compete or interfere with one or more heterologous genes involved in glycosylation that are recombinantly introduced into the host cell) can be deleted or modified in the host cell background (genome) in a manner that makes them inactive/dysfunctional (*i.e.,* the host cell nucleic acids that are deleted/modified do not encode a functional protein or do not encode a protein whatsoever). In certain embodiments, when nucleic acids are deleted from the genome of the host cells provided herein, they are replaced by a desirable sequence, *e.g.*, a sequence that is useful for glycoprotein production.

[0234] Exemplary genes that can be deleted in host cells (and, in some cases, replaced with other desired nucleic acid sequences) include genes of host cells involved in glycolipid biosynthesis, such as *waaL* (*see, e.g.,* Feldman et al., 2005, PNAS USA 102:3016-3021), the lipid A core biosynthesis cluster *(waa),* galactose cluster (*gal*)*,* arabinose cluster (*ara*)*,* colonic acid cluster (wc), capsular polysaccharide cluster, undecaprenol-p biosynthesis genes (e.g. *uppS, uppP*)*,* und-P recycling genes, metabolic enzymes involved in nucleotide activated sugar biosynthesis, enterobacterial common antigen cluster, and prophage O antigen modification clusters like the *gtrABS* cluster.

[0235] The host cells described herein can produce the N-glycosylated carrier proteins described herein. In some embodiments, the N-glycosylated carrier proteins produced by the host cells described herein are antigens, *e.g.*, viral or bacterial antigens that can be used in vaccines. In some embodiments, the N-glycosylated carrier proteins produced by the host cells described herein can be any carrier proteins described herein, wherein said carrier proteins are modified by the host cells described herein so as to possess one or more beneficial characteristics, *e.g.*, the carrier protein is N-glycosylated.

[0236] Certain of the Examples below describe application of methods described herein in Gram-negative *E. coli* host cells; however, any host cells known to those of skill in the art could be used as to produce N-glycosylated carrier proteins, including archea, prokaryotic host cells other than *E. coli,* and eukaryotic host cells.

[0237] Exemplary prokaryotic host cells that can be used in accordance with the methods described herein comprise, without limitation, *Escherichia* species, *Shigella* species, *Klebsiella* species, *Xhantomonas* species, *Salmonella* species, *Yersinia* species, *Lactococcus* species, *Lactobacillus* species, *Pseudomonas* species, *Corynebacterium* species, *Streptomyces* species, *Streptococcus* species, *Staphylococcus* species, *Bacillus* species, and *Clostridium* species.

[0238] In certain embodiments, the host cells described herein comprise a genome into which one or more DNA sequences has been introduced, wherein the DNA sequences encode a protein or comprise an operon/gene cluster involved in the N-glycosylation of proteins. For example, in some embodiments, a host cell described herein comprises a genome into which one or more of the following has been inserted: DNA encoding an N-OST, DNA encoding a glycosyltransferase, DNA encoding a carrier protein, DNA comprising an *rfb* gene cluster, DNA comprising a capsular polysaccharide gene cluster, and/or DNA encoding an epimerase.

[0239] The host cells can include recombinant N-OSTs provided herein or nucleic acids encoding the recombinant N-OSTs provided herein, whereby the recombinant N-OSTs can be from any organism having N-OSTs, including a eukaryotic organism or a prokaryotic organism. In some embodiments, the N-OST protein or N-OST encoding nucleic acid is from the genus *Campylobacter* (*e.g., the pglB* gene from *C. jejuni*).

[0240] The host cells described herein can comprise a glycosyltransferase known in the art or a nucleic acid sequence encoding a glycosyltransferases known in the art. In some embodiments, the glycosyltransferase is a glycosyltransferase described in International Patent Application Publication No. WO 2011/13836].

[0241] In some embodiments, the glycosyltransferase is from a Gram-positive bacterium, *e.g.,* the glycosyltransferase is from *S. aureus.* In some embodiments, the glycosyltransferase is capsular polysaccharide 5 from *S. aureus.* In some embodiments, the glycosyltransferase is capsular polysaccharide 8 from *S. aureus.* In some embodiments, the glycosyltransferase is from a Gram-negative bacterium, *e.g., E. coli.* In some embodiments, the glycosyltransferase is from a eukaryote.

[0242] The host cells described herein can comprise or produce a carrier protein known in the art or comprise a nucleic acid sequence encoding a carrier protein known in the art. The carrier proteins produced by the host cells described herein comprise at least one N-glycosylation consensus sequence, *e.g.*, either the consensus sequence (i) Asn-X-Ser(Thr), wherein X is are independently selected from any amino acid except Pro; or (ii) D/E-X-N-Z-S/T, wherein X and Z are independently selected from any amino acid except Pro. Accordingly, the host cell can comprise DNA sequences encoding an N-glycosylation consensus sequence. The host cell can include any carrier protein known in the art, including the carrier proteins described in Section 5.5. In some embodiments, the carrier protein is an Exotoxin A of *P. aeruginosa* (EPA), including EPA that has been modified to comprise at least one N-glycosylation consensus sequence. In some embodiments, the carrier protein is cholera toxin

B. In some embodiments, the carrier protein is AcrA. In some embodiments, the carrier protein is H1A. In some embodiments, the carrier protein is ClfA.

6.8 Bioconjugates

[0243] The bioconjugates described herein are conjugates between a protein (*e.g.*, any carrier protein described herein; *e.g.,* Section 6.5) and an oligosaccharide or a polysaccharide *(e.g.,* any oligosaccharide or polysaccharide described herein; *see, e.g.,* Section [00201]) prepared in a host cell, wherein host cell machinery links the oligosaccharide or polysaccharide to the protein (*e.g.,* N-links). In some embodiments, the oligosaccharide or polysaccharide is an antigen (*e.g.*, any antigen described herein; *see, e.g.,* Section 6.4). Glycoconjugates can include bioconjugates, as well as sugar antigen *(e.g.,* oligo- and polysaccharides)-protein conjugates prepared by other means, *e.g.*, by chemical linkage of the protein and sugar antigen.

[0244] The recombinant N-OSTs described herein *(see, e.g.,* Section 6.1) can be used to produce host cells that produce bioconjugates comprising an N-glycosylated carrier protein. In some embodiments, provided herein are bioconjugates including a carrier protein N-glycosylated with an antigen (*e.g.*, an oligosaccharide or a polysaccharide) described herein. In some embodiments, the carrier protein is EPA. The bioconjugates described herein can, for example and without limitation, comprise any carrier protein described herein. The bioconjugates described herein can, for example and without limitation, compise any oligosaccharide or polysaccharide described herein.

[0245] In some embodiments, the heterologous *C. jejuni* glycosylated carrier protein is *Pseudomonas aeruginosa* exotoxin (EPA)-*S. dysenteriae* O1 (EPA-O1), EPA-*S. aureus* capsular polysaccharide Type 5 (EPA-CP5) or EPA-*Salmonella enterica (S. enterica)* LT2 (EPA-LT2).

[0246] In some embodiments, provided herein is a bioconjugate including EPA and one or more different oligosaccharides or polysaccharides described herein.

[0247] In some embodiments, provided herein is a bioconjugate including carrier protein conjugated to one or more of *E.coli* O1, O2, O4, O6, O7, O8, O11, 015, 016, 017, 018, O20, O22, O25, O73, O75, and/or O83. In some embodiments, the carrier protein is EPA.

[0248] In some embodiments, provided herein is a bioconjugate including a carrier protein conjugated to one or more different *P. aeruginosa* polysaccharides. In some embodiments, the carrier protein is EPA.

[0249] In some embodiments, provided herein is a bioconjugate comprising a carrier protein conjugated to one or more different *K. pneumonia* polysaccharides. In a specific embodiment, the carrier protein is EPA.

6.9 Methods for Producing a Bioconjugate

[0250] In some embodiments, the recombinant N-OSTs provided herein *(see, e.g.,* Section 6.1) can be used to produce a bioconjugate provided herein *(see, e.g.,* Section 6.8), such as a glycoconjugate. In some embodiments, the recombinant N-OSTs provided herein can be used to produce conjugate vaccines, *i.e.* vaccines that contain a oligosaccharide or polysaccharide (*see, e.g.,* Section 5.4) and a protein antigen of the pathogen that the vaccine is designed against.

[0251] In another aspect, provided herein is a method of producing a bioconjugate including culturing a host cell provided herein *(see, e.g.,* Section 6.7) in a cell culture medium. In some embodiments, the host cell comprises a nucleic acid encoding a recombinant modified N-OST provided herein *(see, e.g.,* Section 6.1 and Section 6.6). In some embodiments, the host cell comprises a nucleic acid encoding a carrier protein described herein *(see, e.g.,* Section 6.5 and Section 6.6). In some embodiments, the carrier protein has one or more N-glycosylation consensus sequence. In some embodiments, the host cell comprises a nucleic acid encoding a glycosyltransferase *(see, e.g.,* Section 6.6 and Section 6.7).

[0252] In some embodiments, the bioconjugate is an N-glycosylated carrier protein. The N-glycosylated carrier protein can comprise a oligosaccharide or polysaccharide component including any oligosaccharide or polysaccharide described herein. *See, e.g.,* Section [00201]. The N-glycosylated carrier protein can comprise any carrier protein described herein. *See, e.g.,* Section 6.5. In some embodiments, the bioconjugate is a natural *C. jejuni* N-glycosylated polypeptide (including a *C. jejuni* oligosaccharide or polysaccharide component and a *C. jejuni* carrier protein). In some embodiments, the bioconjugate is a heterologous *C. jejuni* glycosylated polypeptide (including a polysaccharide component and/or a carrier protein that is not from *C. jejuni).* In some embodiments, the glycosylated polypeptide does not have an N-acetyl sugar at its reducing end. In some embodiments, the glycosylated polypeptide has a galactose at its reducing end.

[0253] In some embodiments, the bioconjugate is produced at a between about 2-fold and about 100-fold, by between about 5-fold and about 80-fold, by between about 10-fold and about 60-fold, by between about 10-fold and about 20-fold or by between about 20-fold and about 40-fold compared faster rate when using a host cell including a recombinant N-OST of this disclosure than when using a host cell including a wild-type form of the recombinant N-OST. In some embodiments, the bioconjugate is produced at a more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold faster rate when using a host cell including a recombinant N-OST of this disclosure than when using a host cell including a wild-type form of the recombinant N-OST.

[0254] In some embodiments, the bioconjugate is produced at a between about 2-fold and about 100-fold, by between about 5-fold and about 80-fold, by between about 10-fold and about 60-fold, by between about 10-fold and about 20-fold or by between about 20-fold and about 40-fold compared greater yield when using a host cell including a recombinant N-OST of this disclosure than when using a host cell including a wild-type form of the recombinant N-OST. In some embodiments, the bioconjugate is produced at a more than 2-fold, more than 3-fold, more than 4-fold, more than 5-fold, more than 6-fold, more than 7-fold, more than 8-fold, more than 9-fold, more than 10-fold, more than 11-fold, more than 12-fold, more than 13-fold, more than 14-fold, more than 15-fold, more than 17-fold, more than 20-fold, more than 25-fold, more than 30-fold, more than 35-fold, more than 40-fold, more than 45-fold, more than 50-fold, more than 60-fold, more than 70-fold, more than 80-fold, more than 90-fold or more than 100-fold greater yield when using a host cell including a recombinant N-OST of this disclosure than when using a host cell including a wild-type form of the recombinant N-OST.

[0255] In some embodiments, between about 1% to about 70%, of between about 3% to about 65%, of between about 5% to about 60%, of between about 10% to about 55%, of between about 15% to about 50%, of between about 20% to about 45%, of between about 20% to about 45%, of between about 25% to about 40%, or of between about 30% to about 35% of carrier protein in the host cell is glycosylated to form the bioconjugate.

[0256] In some embodiments, the at least 1%, at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% of carrier protein in the host cell is glycosylated to form the bioconjugate.

[0257] In some embodiments, the methods further comprise purifying the bioconjugate from the host cell culture. Methods for purifying bioconjugates, such as N-glycosylated carrier proteins, from host cell cultures are known in the art. *See, e.g.,* Jan-Christer Janson, Protein Purification: Principles, High Resolution Methods, and Applications. Wiley; 3 edition (March 22, 2011).

6.10 Analytical Methods

[0258] Various methods can be used to analyze the structural compositions and sugar chain lengths of the bioconjugates or N-glycosylated carrier proteins described herein.

[0259] In one embodiment, hydrazinolysis can be used to analyze glycans. First, polysaccharides are released from their protein carriers by incubation with hydrazine according to the manufacturer's instructions (Ludger Liberate Hydrazinolysis Glycan Release Kit, Oxfordshire, UK). The nucleophile hydrazine attacks the glycosidic bond between the polysaccharide and the carrier protein and allows release of the attached glycans. N-acetyl groups are lost during this treatment and have to be reconstituted by re-N-acetylation. The free glycans are purified on carbon columns and subsequently labeled at the reducing end with the fluorophor 2-amino benzamide (Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal Biochem. 1995 Sep 20;230(2):229-38). The labeled polysaccharides are separated on a GlycoSep-N column (GL Sciences) according to the HPLC protocol of Royle *et al.* (Royle L, Mattu TS, Hart E, Langridge JI, Merry AH, Murphy N, Harvey DJ, Dwek RA, Rudd PM. An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. Anal Biochem. 2002 May 1;304(1):70-90). The resulting fluorescence chromatogram indicates the polysaccharide length and number of repeating units. Structural information can be gathered by collecting individual peaks and subsequently performing MS/MS analysis. Thereby the monosaccharide composition and sequence of the repeating unit could be confirmed and additionally in homogeneity of the polysaccharide composition could be identified. Specific peaks of low molecular weight can be analyzed by MALDI-MS/MS and the result is used to confirm the glycan sequence. Each peak corresponds to a polymer consisting of a certain number of repeat units and fragments thereof. The chromatogram thus allows to measure the polymer length distribution. The elution time is a indication for polymer length, fluorescence intensity correlates with molar abundance for the respective polymer.

[0260] In another embodiment, SDS-PAGE or capillary gel electrophoresis can be used to assess glycans and glycoconjugates. Polymer length for the O antigen glycans which are synthesized here is defined by the number of repeat units that are linearly assembled. This means that the typical ladder like pattern is a consequence of different repeat unit numbers that compose the glycan. Thus, two bands next to each other in SDS PAGE or other techniques that separate by size differ by only a single repeat unit. These discrete differences are exploited when analyzing glycoproteins for glycan size: The unglycosylated carrier protein and the glycoconjugate with different polymer chain lengths separate according to their electrophoretic mobilities. The first detectable repeating unit number (n_1) and the average repeating unit number ($n_{average}$) present on a glycoconjugate are measured. These parameters can be used to demonstrate batch to batch consistency or polysaccharide stability.

[0261] In another embodiment, high mass MS and size exclusion HPLC could be applied to measure the size of the complete glycoconjugates.

[0262] In another embodiment, an anthrone-sulfuric acid assay can be used to measure polysaccharide yields (Leyva A, Quintana A, Sanchez M, Rodriguez EN, Cremata J, Sanchez JC. Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. Biologicals. 2008 Mar;36(2): 134-41. Epub 2007 Nov 26).

(a) *Change in glycosylation site usage*

[0263] To show that the site usage in a specific protein is changed glycosylation site usage can be quantified. Methods to do so are listed below.

[0264] Glycopeptide LC-MS/MS: glycoconjugates are digested with protease(s), and the peptides are separated by a suitable chromatographic method (C18, Hydriphilic interaction HPLC HILIC, GlycoSepN columns, SE HPLC, AE HPLC), and the different peptides are identified using MS/MS. This method can be used with or without previous sugar chain shortening by chemical (smith degradation) or enzymatic methods. Quantification of glycopeptide peaks using UV detection at 215 to 280 nm allow relative determination of glycosylation site usage.

[0265] Size exclusion HPLC: Higher glycosylation site usage is reflected by a earlier elution time from a SE HPLC column. See also (a).
(b) *Homogeneity*

[0266] Glycoconjugate homogeneity (the homogeneity of the attached sugar residues) can be assessed using methods that measure glycan length and hydrodynamic radius.

6.11 Benefits

[0267] The recombinant N-OSTs provided herein (*see, e.g.,* Section 5.1) and methods provided herein (*see, e.g.,* Sections 6.2 and 6.9) of using the recombinant N-OST provided herein (*see, e.g.,* Section 6.1) are of particular commercial importance and relevance, as they allow for rapid, high-yield, large-scale and low-cost fermentation of highly homogeneous bioconjugate preparations (e.g., glycoconjugate preparation or conjugate vaccine preparations). The recombinant N-OSTs provided herein enable an economically viable production of commercially and therapeutically valuable bioconjugates, such as conjugate vaccines. Enzymatic production processes using the recombinant N-OSTs provided herein is expected to yield more homogeneous and repreducible bioconjugate preparations than commonly used chemical synthesis methods. The reproducibility and robustness of biotechnological bioconjugate production methods using the recombinant N-OSTs provided herein, is expected to contribute to a reduction of production costs. The homogeneity of especially biotherapeutic conjugate vaccines is generally believed to affect the clinical safety of drug products.

6.12 Analytical Methods for Testing Benefit

[0268] *Yield.* Yield is measured as carbohydrate amount derived from a liter of bacterial production culture grown in a bioreactor under controlled and optimized conditions. After purification of glycoconjugate, the carbohydrate yields can be directly measured by either the anthrone assay, or ELISA using carbohydrate specific antisera. Indirect measurements are possible by using the protein amount (measured by well known BCA, Lowry, or bardford assays) and the glycan length and structure to calculate a theoretical carbohydrate amount per gram of protein. In addition, yield can also be measured by drying the glycoprotein preparation from a volatile buffer and using a balance to measure the weight.

[0269] *Homogeneity.* Homogeneity means the variability of glycan length and possibly the number of glycosylation sites. Methods listed above can be used for this purpose. SE-HPLC allows the measurement of the hydrodynamic radius. Higher numbers of glycosylation sites in the carrier lead to higher variation in hydrodynamic radius compared to a carrier with less glycosylation sites. However, when single glycan chains are analyzed, they may be more homogenous due to the more controlled length. Glycan length is measured by hydrazinolysis, SDS PAGE, and CGE. In addition, homogeneity can also mean that certain glycosylation site usage patterns change to a broader/narrower range. These factors can be measured by Glycopeptide LC-MS/MS.

7. EXAMPLES

7.1 Example 1: Modeling of $\mathrm{PglB}_{\mathrm{Cj}}$ and *In Silico* Oligosaccharide Binding

[0270] A set of homology models of *C. jejuni* PglB was generated using different homology modelling methods with the experimental C.lari structure as template (PDBid 3RCE). The model generated with the HHpredB method (Söding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 2005:33,W244-8.) was selected because its coordinates was better scored by the QMEAN model quality estimation tool (Benkert P, Biasini M, Schwede T. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics. 2011: 27:343-350). Next, the Mg^{2+} ion and the acceptor peptide were transferred from the C. *lari* structure (PDBid 3RCE). To visualize the orientation of the protein in the cytoplasmic membrane, a phospholipid bilayer model was derived according to the OPM database entry for C. *lari* (Lomize MA, Pogozheva ID, Joo H, Mosberg HI, Lomize AL. OPM database and PPM web server: resources for positioning of proteins in membranes. Nucleic Acids Res. 2012 40:D370-6.). The natural heptasaccharide ligand of *C. jejuni* and the first repeating unit of the *S.enterica* LT2 polysaccharides were parameterized accounting for charges and tautomers, and low energy conformations generated. (Shelley JC, Cholleti A, Frye LL, Greenwood JR, Timlin MR, Uchimaya M: J Comput Aided Mol Des. 2007, (12):681-91). These low energy conformations were then placed into the model, and conformationally sampled (Kolossváry, I.; Guida, W. C. Low-mode Conformational Search Elucidated. Application to C39H80 and Flexible Docking of 9-Deazaguanine Inhibitors to PNP. J. Comput. Chem. 1999, 20, 1671.) The saccharide was defined as freely moving substructure and position constraints have been placed on all protein backbone atoms and on all atoms in a 6 Å radius around the OS. Distance constraints on the CO-N and the N-C1 bond between the first saccharide unit and the ASP-N were applied. A conformational sampling for 1000 steps was set up applying OPLS (Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. J. J. Phys. Chem. B 2001, 105, 6474).), including a full atom water model and a 35 kcalmol^{-1} window to screen possible conformations, which were refined with a similar protocol employing a more narrow energy window of 21 kJmol^{-1}.

[0271] The selection of potential oligosaccharide interacting residues of PglB$_{Cj}$ as targets for mutagenesis required the generation of a homology model which was generated using the experimentally solved structure of C. *lari* PglB (PDBid 3RCE) as template. Binding of the natural *C. jejuni* N-glycan substrate and of the first repeating unit of the heterologous *S. enterica* LT2 polysaccharide to PglB$_{Cj}$ was simulated. The monosaccharide subunit composition of the native oligosaccharide substrate and of the repeating units of two heterologous polysaccharides analysed in this study are shown in FIG. 1. The oligosaccharide structures were conformationally sampled while being covalently attached to the amide nitrogen of the asparagine residue within the acceptor peptide. The conformation of the enzyme and hence the ligand were assumed to be product-like, *i.e.,* just before the release from the active site. The structural models therefore do not consider any of the factors for initial binding of the undecaprenyl-pyrophosphate linked oligosaccharide (OS) substrate. In FIG. 2A, examples of predicted conformations are shown for *C. jejuni* OS and the first repeating unit *of S. enterica* LT2. The overall interactions with the protein residues and the relative orientation in the binding site differed substantially between the two oligosaccharides. Snapshots of thermodynamically favored conformations are shown in FIGs. 2B and 2C. While the natural OS substrate offered a multitude of hydrogen bonding partners to itself and to the surrounding residues of wild-type PglB$_{Cj}$, the LT2 O antigen repeating unit was lacking similar interactions, supporting a mechanistic model in which low transfer efficiency is caused by poor binding of the carbohydrate substrate to the active site. The repeating unit of the LT2 O antigen is composed of four hexoses (FIG. 1) which lack N-acetyl substituents, limiting the possibilities for hydrogen bond formation.

[0272] To select mutagenesis positions within the PglB$_{WT}$ sequence, the generated model was used to identify amino acid side chains that are located in 2.5-4 Å distance to the natural C. *jejuni* N-glycan. The following residues matched this criterion: Y77, S80, S196, N311, Y462, H479 and K522 (FIG.2B). In the case of G476 and G477, the carbonyl oxygen atom of the polypeptide backbone was predicted to be in close distance to the innermost sugar (FIG. 2B).

7.2 Example 2: Mutagenesis of Predicted Sugar-Interacting Residues

[0273] Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* W3110 *waaL* was used as host strain for production of EPA-C. *jejuni* OS and EPA-*Shigella* O1 glycoconjugates, and EPA-CP5 in *in vivo* glycosylation experiments. *S. enterica* sv. Typhimurium SGSC228 which produces LT2 polysaccharides and lacks a functional *waaL* gene was used for production of LT2-EPA. Ultra competent *E. coli* cells were used for initial transformation of constructed plasmid libraries and variants. *E. coli* DH5α was used as standard host for plasmid production and storage.

Table 1. Strains and Plasmids

Strain / plasmid	**Description**	**Selection marker**	**Reference**
Bacterial strains E. coli XL10-Gold	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lat*I*qZ*Δ*M15* Tn*10* (TetR) Amy Cam^R]	Cm^R	Stratagene
E. coli DH5α	K-12 ϕ80d*lacZ*Δ*M15 endA1 recA1 hsdR17*(rK-mK+) *supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 F-*	-	Clontech
E. coli CLM24	W3110 Δ*waaL*	-	(4)
E. coli StGVXN1717	W3110 Δ*waaL* Δ*wecA-wzzE* Δ*rmlB-wecG*::cat	Cm^R	(7)
S. enterica SGSC228 *Plasmids*	sv. Typhimurium LT2; waaL446	-	(5)
pACT3Kan	Medium copy number vector for IPTG-inducible expression; *lacI*, P_{tac}, ori: pACYC184/p15a	Kan^R	(11)
pEXT21	Low copy number vector for IPTG-inducible expression; *lacI*, P_{tac}, ori: IncW	Sp^R	(47)
pACYC(pgl_{mut})	C. *jejuni* heptasaccharides, constitutive expression; *pgl* operon of *C. jejuni* with inactive PglB variant W458A-D459A (PglB_{mut}), ori: pACYC184/p15a	Cm^R	(2)
pGVXN64	*Shigella dysenteriae* O1 LPS polysaccharides, constitutive expression; ori: IncPa	Tet^R	(8)
pGVXN115	PglB_{mut}, IPTG inducible expression; pEXT21 vector (ori: IncW)	Sp^R	(8)
pGVXN150	*Pseudomonas aeruginosa* exotoxoid A (EPA) with 2 engineered N-glycosylation sites, L-arabinose inducible expression; N-terminal ssDsbA signal peptide for secretion to periplasm and C-terminal 6H tag, ori: pBR322	Amp^R	(7)
pGVXN393	*Staphyloccus aureus* CP5 capsular polysaccharides, constitutive expression; ori: IncPa	Tet^R	(7)
pGVXN408	Inactive *C. jejuni* PglB-HA W458A-D459A (PglB_{mut}), IPTG inducible expression; pACT3Kan vector	Kan^R	(11)

Strain / plasmid	**Description**	**Selection marker**	**Reference**
pGVXN925	*C. jejuni* PglB with C-terminal hemagglutinin (HA) tag, codon-optimized for *E. coli*; high copy number cloning vector pUC57	Amp^R	This study
pGVXN970	wild-type, untagged $PglB_{Cj}$, codon-optimized for *E. coli,* IPTG inducible expression; pEXT21 vector	Sp^R	This study
pGVXN1049	*C. jejuni* PglB-HA, codon-optimized for *E. coli,* IPTG inducible expression; pACT3Kan vector	Kan^R	This study
pGVXN1050	wild-type, untagged $PglB_{Cj}$, codon-optimized for *E. coli,* IPTG inducible expression; pACT3Kan vector	Kan^R	This study
pGVXN1217	$PglB_{Cj}$ N311V, derivative of pGVXN970	Sp^R	This study
pGVXN1413	wild-type $PglB_{Cj}$, semi-constitutive expression; size-reduced pACT3Kan vector without *lacI*	Kan^R	This study
pGVXN1415	$PglB_{Cj}$ N311V-A669V, semi-constitutive expression; size-reduced pACT3Kan vector without *lacI,* isolated from a saturation mutagenesis library of pGVXN1050	Kan^R	Ihssen *et al.*, in preparation
pGVXN1418	$PglB_{Cj}$ N311V, derivative of pGVXN1415	Kan^R	This study
pGVXN1942	$PglB_{Cj}$ S80R-Q287P-N311V, isolated from a shuffled library, derivative of pGVXN1418	Kan^R	This study

[0274] Codon optimized PglB was expressed from low copy plasmid pEXT21 and obtained from a gene synthesis service company (Genescript). A template plasmid for construction of *pglB* libraries was constructed by PCR-subcloning of codon-optimized *pglB-HA* from pGVXN925 into pACT3Kan, using restriction sites *Kpn*I and *BamH*I (pGVNX1049), followed by insertion of a TAA stop codon in front of the sequence encoding the HA peptide tag by QuikChange (pGVNX1050). A size-reduced pACT3Kan derived template plasmid pGVXN1413 which lacks the *lacI* repressor gene (semi-constitutive expression of PglB) was constructed by ligation of a *Kpn*I-*BamH*I fragment of pGVXN1050 (encoding wild-type PglB) into the *BamH*I-*Kpn*I vector backbone fragment of pGVXN1415. Size reduced template plasmid pGVXN1418 (PglB N311V) for second round libraries was constructed by ligation of an *AscI-BamH*I fragment of pGVXN1050 (last third of wt *pglB* gene) into a *BamH*I-*AscI* fragment of pGVXN1415. All plasmids were validated by DNA sequencing.

[0275] Appropriate antibiotics were added to all growth media to ensure plasmid maintenance

(Ampicillin, Amp: 100 mg L^{-1}, Chloramphenicol, Cm: 10 mg L^{-1}, Kanamycin, Kan: 30 mg L^{-1}, Spectimomycin, Sp: 80 mg L^{-1}, Tetracyclin, Tet: 20 mg L^{-1}).

[0276] Mutagenic primers and sequencing services were obtained from Microsynth (Balgach, Switzerland). PglB variants and libraries were constructed by QuikChange using pGVXN1050, pGVXN1415 or pGVXN1418 as template. Desired mutations were verified by sequencing.

[0277] Only sequences of forward primers of each primer pair is given, respective reverse complement sequences were used for the reverse primers. Degenerate codons at mutated position(s) are underlined. Saturation mutagenesis of N311: 5'-GC TTC ATG TAC TTC AAC GTT NNK CAG ACG ATC CAA GAA GTG G-3' (SEQ ID NO:3), saturation mutagenesis of Y77: 5'-CAT CAG CCG AAC GAT CTG AGT NNK TAC GGT AGC TCT CTG TCC G-3'(SEQ ID NO:4), four amino acid (Ala, Ser, Cys, Gly) randomization of G476-G477 5'-C GAT GTT AAA ACG CTG GTC GAC KST KST AAA CAC CTG GGC AAG G-3' (SEQ ID NO:5), saturation mutagenesis of S80 5'-CG AAC GAT CTG AGT TAT TAC GGT NNK TCT CTG TCC GCG CTG ACC-3' (SEQ ID NO:6), saturation mutagenesis of Q287 5'-GGT GTT GAT CCG ATT CTG TAC NNK CTG AAA TTT TAT ATC TTC CGC TCA G-3' (SEQID NO:7), saturation mutagenesis of L288 5'-GTT GAT CCG ATT CTG TAC CAG NNK AAA TTT TAT ATC TTC CGC TCA GAT G-3' (SEQ ID NO:8), saturation mutagenesis of K289 5'-GAT CCG ATT CTG TAC CAG CTG NNK TTT TAT ATC TTC CGC TCA GAT GAA TCG-3' (SEQ ID NO:9), saturation mutagenesis of F290 5'-CCG ATT CTG TAC CAG CTG AAA NNK TAT ATC TTC CGC TCA GAT GAA TCG-3' (SEQ ID NO: 10), saturation mutagenesis of Y291 5'-CG ATT CTG TAC CAG CTG AAA TTT NNK ATC TTC CGC TCA GAT GAA TCG-3' (SEQ ID NO:11), saturation mutagenesis of R294 5'-G TAC CAG CTG AAA TTT TAT ATC TTC NNK TCA GAT GAA TCG GCA AAC CTG-3' (SEQ ID NO:12). First round libraries were constructed using wild-type PglB plasmids pGVXN1050 or pGVXN1413 as template. Second round libraries were constructed using pGVXN1418 (PglB N311V) or pGVXN1930 (PglB-HA N311V) as template. Initial mutant libraries were based on pGVXN1050 as template plasmid. However, it was found that such libraries repeatedly yielded variant plasmids with a 2.1 kB reduced vector backbone due to a recombination event at a repetitive sequence present in the original pACT3 sequence. The size reduction lead to loss of the *lacI* repressor gene, which in turn facilitated semi-constitutive expression of PglB and a two-fold increase in EPA-CP5 levels (published elsewhere). In order to avoid such unwanted recombination, pGVXN1413, pGVXN1418 and pGVXN1930 were used as template in later libraries.

[0278] A shuffled library of neutral and slightly beneficial second round variants was constructed with the Multi Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene). A mix of three oligonucleotides was used, primer 5'- CAT CAG CCG AAC GAT CTG AGT YMT TAC GGT MGT TCT CTG TCC GCG CTG AC-3' (SEQ ID NO:13) targeting the Y77 region and a 4:1 molar ratio mix of primers 5'- C GGT GTT GAT CCG ATT CTG TAC MVG WTK MAK TTT TAT ATC TTC CGC TCA GAT GAA TCG -3' and 5'- C GGT GTT GAT CCG ATT CTG TAC MVG WTK CGT TTT TAT ATC TTC CGC TCA GAT GAA TCG -3' (SEQ ID NO:14) targeting the EL5 region. Improved PglB variant N311V (pGVXN1418) was used as template.

[0279] Only libraries with less than 20% of wild-type clones were used for screening. Plasmid libraries were produced by resuspending at least 1000 XL10-Gold colonies (5000 colonies for the shuffled library) in phosphate buffered saline (PBS), followed by plasmid purification with a standard mini-prep kit. Plasmid libraries were transformed into *E. coli* and *S. enterica* expression strains using standard electroporation procedures.

[0280] Mutant libraries and individual variant plasmids were screened in 96-deep well plates as described previously, except that the concentration of IPTG added at induction was reduced to 30 µM in order to reduce inclusion body formation. PglB variant plasmids were isolated from expression strains by retransformation of plasmid preps in chemically competent *E. coli* DH5α and selection for Kanamycin resistance only. Mutations were characterized by Sanger sequencing of purified plasmids, employing two overlapping reads. Chemically or electrocompetent *S. enterica* SGSG228 (pGVXN150), *E. coli* St1717 (pGVXN150, pGVXN393), and *E. coli* W3110 *waaL* (pGVXN64, pGVXN150) were used as host strains for LT2-EPA, CP5-EPA and O1-EPA DWP-ELISA screenings, respectively.

[0281] Host strains for EPA-CP5, EPA-LT2 and EPA-O1 production in shake flask experiments were similar to DWP experiments. *E. coli* W3110 *waaL* (pACYC(pgl_{mut}), pGVXN150) was used as host strain for EPA-*Cj* OS production. The kinetics of glycoprotein formation were recorded by preparing biomass-normalized periplasmic protein extracts at regular intervals after induction, followed by sandwich ELISA. Triplicate preculture tubes with LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} and 5 g L^{-1} NaCl) were inoculated with individual single colonies from fresh streak-out or transformation plates and incubated overnight at 37°C and 160 rpm. Triplicate Erlenmeyer flasks with 50% v/v of LB-M9 medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 12.8 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$, 3.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl, 2 mM $MgSO_4 \cdot 7H_2O$ and 0.1 mM $CaCl_2$) were inoculated 1:50 from tube pre-cultures and incubated at 37°C and 160 rpm. At an OD_{600} of 0.5, 1 mM IPTG and 4 g L^{-1} L-arabinose were added for induction and stirrer speed was reduced to 100 rpm. In the case of the *Salmonella* host strain, EPA/EPA-LT2 degradation was observed in LB-M9 shake flasks cultures after overnight induction. Degradation could be prevented by using a high strength complex medium (2YT, 10 g L^{-1} yeast extract, 14 g L^{-1} tryptone, M9 salts), reducing OD_{600} at induction to 0.3-0.4 and switching to static incubation (fully anaerobic growth) after induction.

[0282] Periplasmic extracts were prepared. Extracts were diluted 1000-to 20'000-fold in PBS with 1% w/v dry milk and analysed by sandwich ELISA. Only dilutions yielding non-saturated ELISA signals (Absorbance at 450 nm below 1.0) were used for data analysis. For purification of hexahistidine-tagged proteins, periplasmic extracts of triplicate overnight shake flask cultures were pooled and Ni-affinity chromatography was performed according to standard protocols using HiTrap FF columns (GE Healthcare). SDS-PAGE and Coomassie staining were carried out using standard methods. The relative combined intensity of EPA-LT2 glycoforms was quantified with the software ImageJ (imagej.nih.gov).

[0283] Extracts of periplasmic proteins were diluted appropriately and analysed by sandwich ELISA in 96-well plates. The capture antibody for all ELISA analyses was protein G purified goat-anti EPA antiserum. Rabbit anti-*S. aureus* CP5, rabbit anti-*Salmonella* O:5/O:4 (Staten Serum Institute, Denmark), rabbit anti-*Shigella* O1 and rabbit anti-C. *jejuni* were used for detection of EPA-linked oligo- and polysaccharides. Horseradish peroxidase (HRP) coupled goat anti-rabbit IgG (Bio-Rad, Reinach, Switzerland) and Ultra-TMB substrate (Thermo-Scientific/Pierce) were used for ELISA development. The HRP reaction was stopped by addition of 2M H_2SO_4 and absorbance at 450 nm (ELISA signal) was measured against air with a plate reader. Appropriate development times were chosen so that signal saturation could be avoided (maximal abs. 450 nm ≤ 1.0). Signals were background-corrected by subtracting average absorbance values of samples derived from isogenic control strains expressing PglB_{mut}. Western blot analysis of periplasmic and total cell proteins was performed as described previously. EPA and PglB-HA were detected by rabbit anti-EPA (Sigma-Aldrich, Buchs, Switzerland) and rabbit anti HA (Sigma-Aldrich) primary antibodies, respectively. Oligo- and polysaccharides conjugated to EPA were detected with the same antibodies as used for ELISA.

[0284] In the PglB_{Cj} model with *C. jejuni* OS the amide group of N311 formed a direct hydrogen bond with the C6 hydroxyl group of the second monosaccharide, *i.e.,* GalNAc (counted from the reducing end) (FIG. 2A). A *pglB* plasmid where N311 was randomly changed to all other 19 amino acids was transformed into a *Salmonella* strain expressing LT2 polysaccharides and a non-toxic form of *Pseudomonas aeruginosa* exotoxin (EPA). Screening in a 96 well plate using glycoprotein specific ELISA yielded three clones that showed significantly improved glycosylation efficiency compared to wild-type PglB (FIG. 3A). In all of them, residue N311 was mutated to valine (codons: 2x GTT, 1x GTG).

[0285] The same library was then transformed into *E. coli* cells expressing the *S. aureus* capsular polysaccharide Type 5 (CP5) and EPA. Seven clones were identified that showed an improved glycosylation efficiency compared to wild-type PglB by measuring CP5-EPA productivity by ELISA (FIG.3B). Six of them harbored the amino acid substitution N311V (codons GTG and GTT) and one exhibited mutation N311I (codon ATT). The high fraction of active clones (≈ 80%) indicates that N311 is highly mutation tolerant, which is in agreement with considerable variability at this position in homologous N-OST sequences (FIG. 4).

[0286] Another amino acid localized in close proximity to the oligosaccharide binding site was mutagenized as above. Y77 resides in a periplasmic loop of the transmembrane domain and may interact with oligosaccharides via water mediated and direct hydrogen bonds according to our model (Fig.2A). The residue was found to be highly mutation-tolerant (80-90% CP5-EPA producing clones in a saturation mutagenesis library); again corresponding to a high degree of variability in homologous protein sequences (FIG. 4). Only neutral amino acid substitutions (Y77L, Y77F) were found, but no improved variants were identified.

[0287] Residues Y462, G476, G477 and H479 were also modeled to be in close distance to bound natural oligosaccharide in the $PglB_{Cj}$ model (FIG.2A); however, they are highly conserved in bacterial N-OST. In spite of restricting changes to naturally occurring amino acid substitutions, CP5-EPA glycoconjugate signals were reduced by 50-90% in Y462, G476, G477 and H479 $PglB_{Cj}$ variants, with the notable exception of G476P and H479N which were found to be neutral mutations (FIG. 5). Random combinations of the small amino acids alanine, serine, cysteine and glycine at G476-G477 all led to reduced or abolished CP5-EPA production.

[0288] In summary, from this first round of mutagenesis of amino acids in close proximity of the oligosaccharide binding site of PglB , N311 was identified as the position with the highest impact on improving glycosylation yields. In addition, Y77 was also identified as mutation tolerant.

7.3 Example 3: Effect of N311V on Glycoprotein Formation Rates

[0289] The effect of $PglB_{Cj}$ variant N311V on *in vivo* glycosylation rates for different oligo-/polysaccharide substrates was analysed in shake flask culture (FIG. 6). $PglB_{Cj}$ N311V and PglBwt were expressed from a low copy number vector. Cells expressing the mutant N-OST yielded 8-fold more LT2-EPA after overnight induction (FIG. 6A). The improvement factors after 2h and 4h of induction were 22- and 11-fold, respectively. The initial rate of CP5-EPA formation was increased by a factor of 5.1 (FIG. 6B). Initial rates of O1-EPA formation were also increased two-fold in mutant N311V (FIG. 6C), although this polysaccharide substrate was not used in library screenings. By contrast, no significant effect was found for *in vivo* glycosylation of EPA with the natural *C. jejuni* OS substrate of PglB. The increase in ELISA signals over time and the beneficial or neutral effect of N311V corresponded to Western blot results for exemplary periplasmic protein samples (FIG. 7).

[0290] To analyze the cause of the beneficial effect of N311V production we constructed wild-type PglB and PglB N311V variants with a C-terminal hemeagglutinin (HA) peptide tag. This allowed us to follow expression levels of PglB during the experiments. PglB-HA specific bands in biomass-normalized whole cell protein samples originating from mutants were less intense and more variable than those from wild-type PglB (FIG. 8A). Degradation products corresponding in size to the C-terminal periplasmic domain appeared after induction, indicating a destabilizing effect of the mutation. In spite of the apparent negative effect on PglB stability, EPA-CP5 production followed by ELISA was again significantly increased in cells expressing $PglB_{Cj}$ N311V (FIG. 8B).

7.4 Example 4: Further Rounds of Mutagenesis and Screening

[0291] Following the principle of iterative saturation mutagenesis, $PglB_{Cj}$ N311V was used as template for randomization of Y77 and S80. The latter residue also varies between PglB homologues (FIG. 4) and faces the modeled oligosaccharide substrate binding site just above the position where the external loop EL1 protrudes from the membrane (FIG. 2). Both Y77 and S80 were found to be highly mutation tolerant, with 70-80% active clones when screened for LT2-EPA production (Table 1). The ten clones with the highest ELISA signals were sequenced, and Y77 was changed to diverse amino acids, with a bias towards residues with basic side chains (Table 2). In the NNK library randomizing S80, variant S80R was dominating in the top-performing clones (Table 2).

Table 2. Mutation tolerance of PglB_{Cj} residues mutated in second round saturation mutagenesis libraries and amino acid substitutions identified in the 10 clones with the highest LT2-EPA ELISA signals. Clones were counted as active when background-corrected ELISA signals reached more than 10% of the average value of N311V control wells.

PglB_{Cj} residue	**Fraction of active clones in NNK library**	**Mutations identified in top 10 clones**
Y77	86%	Y77H (2x)
		Y77T
		Y77W
		Y77R
		Y77K (2x)
		Y77A
		Y77G
S80	81%	S80R (8x)
		S80H
Q287	65%	Q287P (4x)
		Q287K (2x)
		Q287R
L288	61%	L288M (2x)
		L288F
		L288I
		L288I
K289	78%	K289R (4x)
		K289N (2x)
		K289Q (2x)
F290	16%	none (all wt)
Y291	3.9%	none (all wt)
R294	22%	R294K

[0292] The $PglB_{Cj}$ $Q_{287}LKFYxxR_{294}$ motif within the N-terminal part of EL5 is highly conserved in PglB sequences of *Campylobacter* species, but not in N-OST of more distantly related species (FIG. 9). Due to the observation that the innermost two sugar subunits of N-linked glycans of *Campylobacter* species are similar (1^{st} 2,4-diacetamido-2,4,6-trideoxyhexose, 2^{nd} N-acetyl-hexosamine), it was hypothesized residues of the *Campylobacter*-specific $Q_{287}LKFYxxR_{294}$ motif may influence oligosaccharide specificity. Saturation mutagenesis libraries were generated at these positions with improved variant N311V as template. When screened in the host strain for LT2-EPA production, a clear difference was observed for the first and second part of the motif (Table 2). While saturation mutagenesis of Q287, L288 and K289 yielded 60-80% active clones, the adjacent residues F290, Y291 and R294 were highly mutation-sensitive. The 10 top performing clones of the Q287, L288 and K289 libraries exhibited non-random amino acid substitutions (Table 2). Proline and the positively charged amino acids lysine and arginine were overrepresented at position Q287. At L288 alternative hydrophobic residues (M, I, F or C) were found exclusively. A bias for residues with either amide (Q, N) or positively charged side chains (R) was observed at position K289 (Table. 2).

[0293] In a final step, the neutral and slightly beneficial mutations found for residues Y77, S80, Q287, L288 and K289 were shuffled. When 720 clones of this library were screened for LT2-EPA production, numerous positive outliers were identified, of which an example is given in FIG. 10A. Clones with at least 2.5-fold increased ELISA signals compared to the average signal of template control clones on the same plate were sequenced (n = 14). S80R was detected in 79% (n = 11), Q287P in 43% (n = 6) and Y77H in 29% (n = 4) of these clones, respectively. The double mutants Y77H-N311V and S80R-N31 IV were found two and four times, respectively. Q287P occurred only in combination with either Y77H or S80R. Y77S, L288I, L288F, K289R and K289Q were found once or twice in combination with the more frequently observed mutations, indicating that these amino acid substitutions were neutral.

[0294] Representative improved variant plasmids were retransformed into the EPA-LT2 expression strain and rescreened in triplicate DWP minicultures (Fig. 10B). A significant additive beneficial effect was verified for both Y77H (2.1-fold to N311V) and S80R (1.8-fold to N311V). When combined with S80R-N311V, Q287P lead to a further increase in EPA-LT2 ELISA signals by a factor of 1.7, resulting in a total improvement factor of 15 relative to wild-type PglB in overnight induced DWP cultures (FIG. 10B). The additive improvement of EPA-LT2 formation was also observed when Ni-NTA affinity chromatography purified material from shake flask cultures was analyzed by SDS-PAGE and Western blot (FIG. 10C). Glycoforms hybridized with antibodies specific for *Salmonella* serotypes O:4 and O:5, detecting the branching abequose sugar within the LT2 polysaccharide and its O-acetylated form, respectively. Using image quantification software, the combined intensity of glycoform bands (> 80 kDa) was increased by factors of 9 and 16 for $PglB_{Cj}$ variants N311V and S80R-Q287P-N31 1V, respectively.

[0295] In conclusion, it has been demonstrated that recombinant N-OSTs with modified substrate specificities were successfully identified. Specifically, N-OSTs were identified that can conjugate carrier proteins with oligosaccharides or polysaccharides lacking N-acetyl groups in the monosaccharide unit at the non-reducing ends of the oligosaccharides or polysaccharides. Advantageously, the identified N-OSTs comprise certain amino acid substitutions and allow for the production of medically relevant glycovaccines at increased rates and with increased yields compared to wild-type N-OSTs.

7.5 Example 5: Mutagenesis of PglB *C. jejuni* residues K482 and D483

[0296] Non-conserved PglB$_{Cj}$ residues K482 and D483 were simultanously randomized to 12 non-redundant, chemically diverse amino acids (S, N, I, V, D, G, F, Y, C, L, H, R) by QuikChange using forward oligonucleotide primer 5'- GTA GAT GGT GGA AAG CAT TTW GGT NDT NDT AAT TTT TTC CCT TCT TTT GCT TTA AGC -3' and reverse primer 5'-GCT TAA AGC AAA AGA AGG GAA AAA ATT AHN AHN ACC WAA ATG CTT TCC ACC ATC TAC -3' (mutated codons underlined; N = A, T, G or C; D = A, T or G; W = A or T). Medium copy-number, IPTG inducible plasmid pGVXN407 encoding the gene sequence for wild-type PglB with C-terminal HA tag was used as template. The quality of the library was verified by sequencing of 20 randomly picked clones. All desired nucleotides were detected at the mutated codons and the proportion of clones with wild-type *pglB* sequence was below 15%. The library, designated Fa, was transformed into the expression strain *E. coli* St1717 (pGVXN150, pGVXN393) and screened in 96 deep-well plates as described previously (Ihssen et al., 2012, BMC Biotechnolgy 12:67). In total 801 clones were screened, exemplary screening results are shown in Figure 10. Clone Fa8_G10 (marked with a circle in FIG. 10) exhibited significantly increased glycoprotein-specific ELISA signals when rescreened in 8 replicate wells and was found to harbor the double mutation PglB$_{Cj}$ K482R-D483H (Table 3). The amino acid changes K482R-D483H were introduced into the wild-type *pglB* sequence of low copy number plasmid pGVXN114 by QuikChange, resulting in plasmid pGVXN635. The wild-type and variant plasmids were transformed into expression strain *E. coli* St1717 (pGVXN150, pGVXN393) and production of CP5-EPA was analysed in triplicate shake flask experiments as described previously (Ihssen et al., 2012, BMC Biotechnolgy 12:67). The double mutant PglB$_{Cj}$ K482R-D483H facilitated a 1.2 to 2.0-fold increase in CP5-EPA levels as determined by sandwich ELISA (FIG. 11).

Table 2. Rescreening and sequencing of top-performing clones of library Fa with randomized residues PglB$_{Cj}$ K482 and D483.

Clone	**ELISA absorbance CP5-EPA (450 nm)** fold difference to wild-type plasmid pGVXN407 (average value of n = 8 replicate wells)	**Significance niveau for increase compared to wild-type PglB** (T-test P value)	**Sequencing results** (amino acid substitutions in PglB$_{Cj}$)	
Fa8_G10	1.63	0.0025	**K482R**	**D483H**
Fa6_D10	1.43	0.002	L480F	**K482R**
			D483F	
Fa7_C7	1.22	0.096	L480F	K482S

Clone	**ELISA absorbance CP5-EPA (450 nm)** fold difference to wild-type plasmid pGVXN407 (average value of n = 8 replicate wells)	**Significance niveau for increase compared to wild-type PglB** (T-test P value)	**Sequencing results** (amino acid substitutions in PglB$_{Cj}$)	
			D483H	

[0297] Wild-type PglB$_{Cj}$ and K482R-D483H variant plasmids pGVXN114 and pGVXN635, respectively, were transformed into the expression strain *E. coli* St2457 (pGVXN570, pGVXN393) which expresses *S. aureus* α-hemolysin (Hla) with an engineered glycosylation site. Strains were inoculated from overnight pre-cultures to an OD_{600} of 0.1 in 1 liter flask cultures (SOB medium + Chloramphenicol, Ampicillin, Tetracycline and Spectinomycin) and incubated at 37°C with shaking. At an OD_{600} of about 1.0 expression of PglB and Hla was induced by adding 1 mM IPTG and 2 g L^{-1} L-arabinose, respectively. In a control experiment IPTG was omitted. Induced cultures were incubated with shaking overnight at 37°C until harvest. The total incubation time was about 23 h.

[0298] In the experiment with induction, 1200 OD were harvested for both strains, while in the ex-periment without PglB induction, a total of 1500 OD were harvested from the overnight cultures. After washing pelleted cells once with 0.9% NaCl, cells were resuspended to an OD_{600} of 50 in resuspension buffer (25% sucrose, 10 mM EDTA, 200 mM Tris·HCl, pH 8.5). The cell suspensions were rotated for 20 min. Cells were then separated by centrifugation and resuspended to an OD_{600} of 50 in osmotic shock buffer (10 mM Tris·HCl, pH 8.5) and incubated for 30 min under gentle agitation. After another centrifugation step, 20 mM $MgCl_2$, 0.5M NaCl, 10 mM imidazole and 30 mM Tris·HCl (pH 8.0) were added to the supernatant. His-tagged Hla and CP5-Hla were purified from the supernatant (= osmotic shock fluid) following standard procedures. Fractions (1 mL) covering the A_{280} elution peak of all four experiments were analyzed by SDS-PAGE (Coomassie staining), anti-CP5 Western blot and anti-His Western blot (Fig. 12). CP5-specific bands (box with broken lines) were stronger for the strain expressing PglB$_{Cj}$ K482R-D483H (pGVXN635) than for the strain expressing wild-type PglB$_{Cj}$ (pGVXN114). An enhancement was found both in IPTG-induced and non-induced shake flask cultures. The overall intensity of CP5-specific bands in the molecular mass range 50-110 kDa was quantified with ImageJ software (http://imagej.nih.gov/ij/). The Grey value of the local background was substracted. HisTrap eluates of the strain expressing PglB$_{Cj}$ K482R-D483H contained 2.0-fold more CP5-Hla than that of the strain expressing wild-type PglB$_{Cj}$.

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SEQUENCE LISTING

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<110> GLYCOVAXYN AG Kowarik, Michael Thomas Ihssen, Julian Haas, Jurgen Thony-Mey, Linda Christiane Franz Schwede, Torsten

<120> COMPOSITIONS AND METHODS FOR PROTEIN GLYCOSYLATION

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Patentkrav

1. Rekombinant N-oligosaccharyltransferase, der er PglB af *Campylobacter jejuni* (PglB$_{Cj}$) eller PglB af *Campylobacter lari* (PglB$_{Cl}$), hvor den rekombinante N-oligosaccharyltransferase (N-OST) detekterbart kan forbinde et oligosaccharid eller polysaccharid, der mangler et N-acetylsukker ved den reducerende ende, med et bærerprotein ved en N-glykosylerings-konsensussekvens, hvor en eller flere aminosyrer udvalgt fra gruppen bestående af Y77, S80, S196, N311, Y462, K482, D483 og G477 af PglB$_{Cj}$ er modificeret.

2. Rekombinant N-oligosaccharyltransferase ifølge krav 1, hvor den rekombinante N-oligosaccharyltransferase omfatter en modificering i en eller flere aminosyrer, hvis sidekæder befinder sig inden for en afstand på 2,5-4,0 Å fra en af de tre terminale monosaccharidenheder ved den reducerende ende af oligosaccharid- eller polysaccharidbestanddelen af et bundet N-glykosyleret bærerprotein i en strukturel model af et kompleks af den rekombinante N-oligosaccharyltransferase og det N-glykosylerede bærerprotein.

3. Rekombinant N-oligosaccharyltransferase ifølge et af kravene 1 til 2, hvor den rekombinante N-oligosaccharyltransferase omfatter modificering i to eller flere aminosyrer.

4. Rekombinant N-oligosaccharyltransferase ifølge et af kravene 1 til 3, hvor mindst en af den ene eller flere aminosyrer befinder sig i et periplasmatisk sløjfe af et transmembrandomæne af den rekombinante N-oligosaccharyltransferase.

5. Rekombinant N-oligosaccharyltransferase ifølge krav 1, hvor den rekombinante N-oligosaccharyltransferase yderligere omfatter en mutation i en eller flere aminosyrer i et QLKFYxxR-motiv.

6. Rekombinant N-oligosaccharyltransferase ifølge krav 5, hvor N311 af PglB$_{Cj}$ er modificeret.

7. Rekombinant N-oligosaccharyltransferase-mutant ifølge et af kravene 1-6, hvor den rekombinante PglB$_{Cj}$ yderligere omfatter en modificering i en eller flere aminosyrer udvalgt fra gruppen bestående af Y77 og S80.

8. Rekombinant N-oligosaccharyltransferase-mutant ifølge et af kravene 1-7, hvor den rekombinante PglB$_{Cj}$ yderligere omfatter en aminosyremodificering i en eller flere aminosyrer af Q287LKFYxxR294-motivet af PglB$_{Cj}$.

9. Rekombinant N-oligosaccharyltransferase-mutant ifølge krav 8, hvor den rekombinante PglB$_{Cj}$ omfatter en aminosyremodificering i en eller flere aminosyrer udvalgt fra gruppen bestående af Q287, L288, K289 og R294.

10. Nukleinsyre, der koder for en rekombinant N-oligosaccharyltransferase ifølge et af kravene 1 til 9.

11. Værtscelle omfattende en rekombinant N-oligosaccharyltransferase ifølge et af kravene 1 til 9.

12. Fremgangsmåde til at producere et biokonjugat omfattende at dyrke en værtscelle ifølge krav 11 i et cellekulturmedium.

13. Fremgangsmåde ifølge krav 12, yderligere omfattende at oprense biokonjugatet fra værtscellekulturen.

DRAWINGS

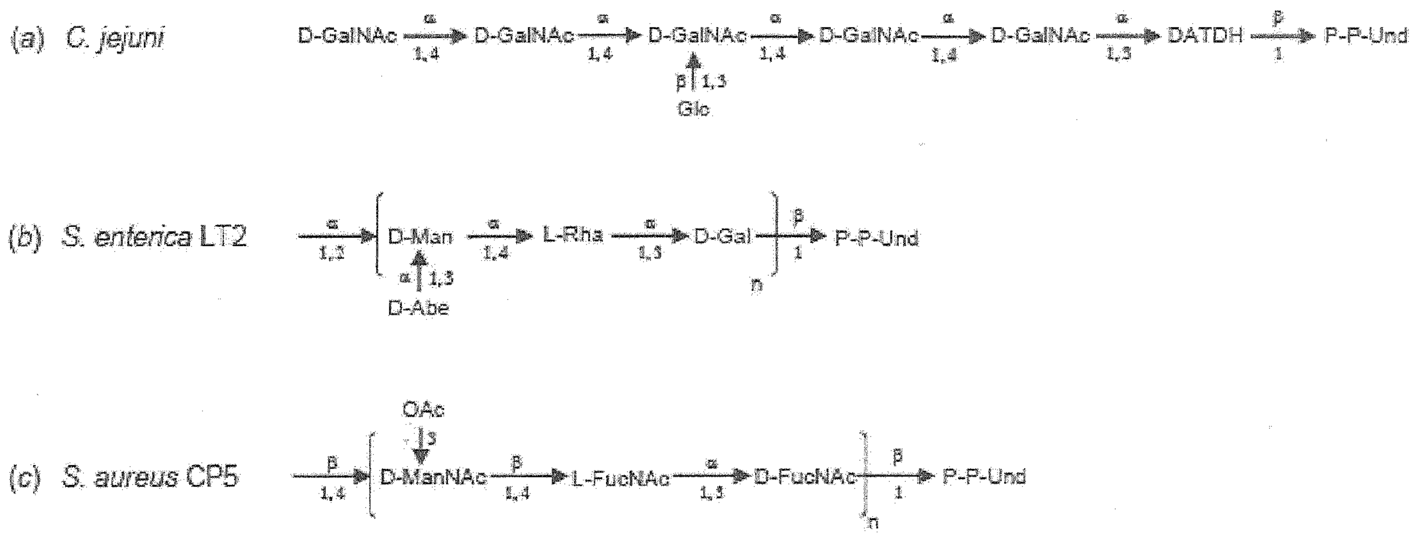


FIG. 1

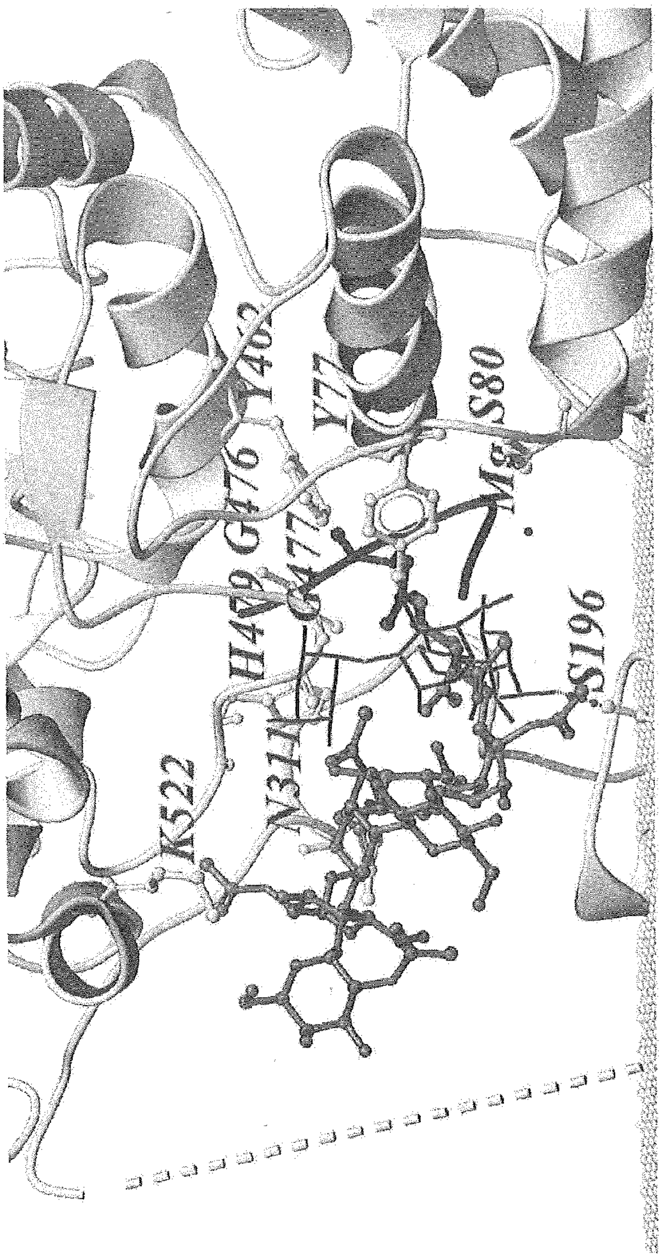


FIG. 2A

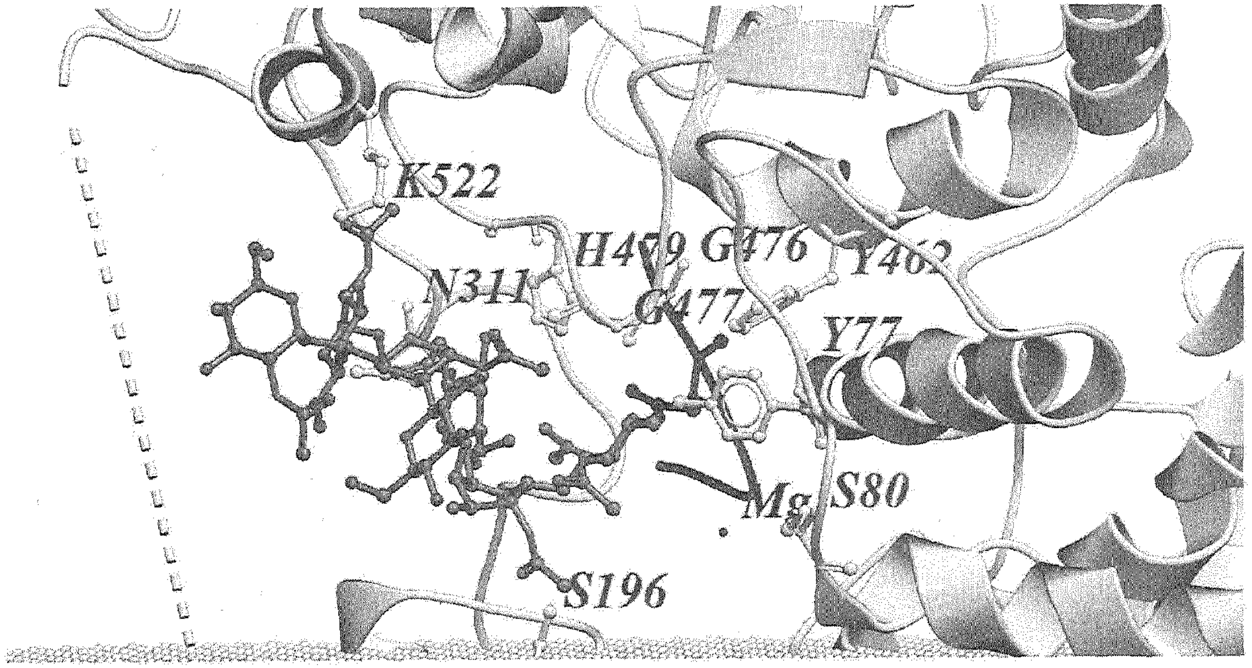


FIG. 2B

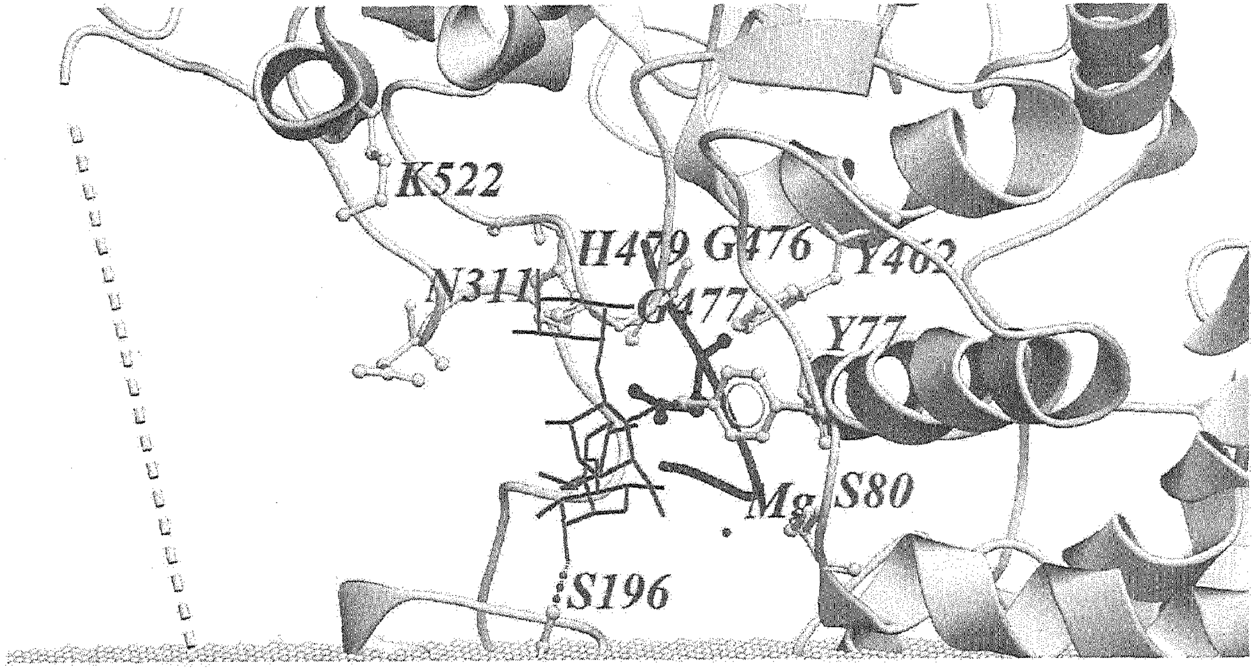


FIG. 2C

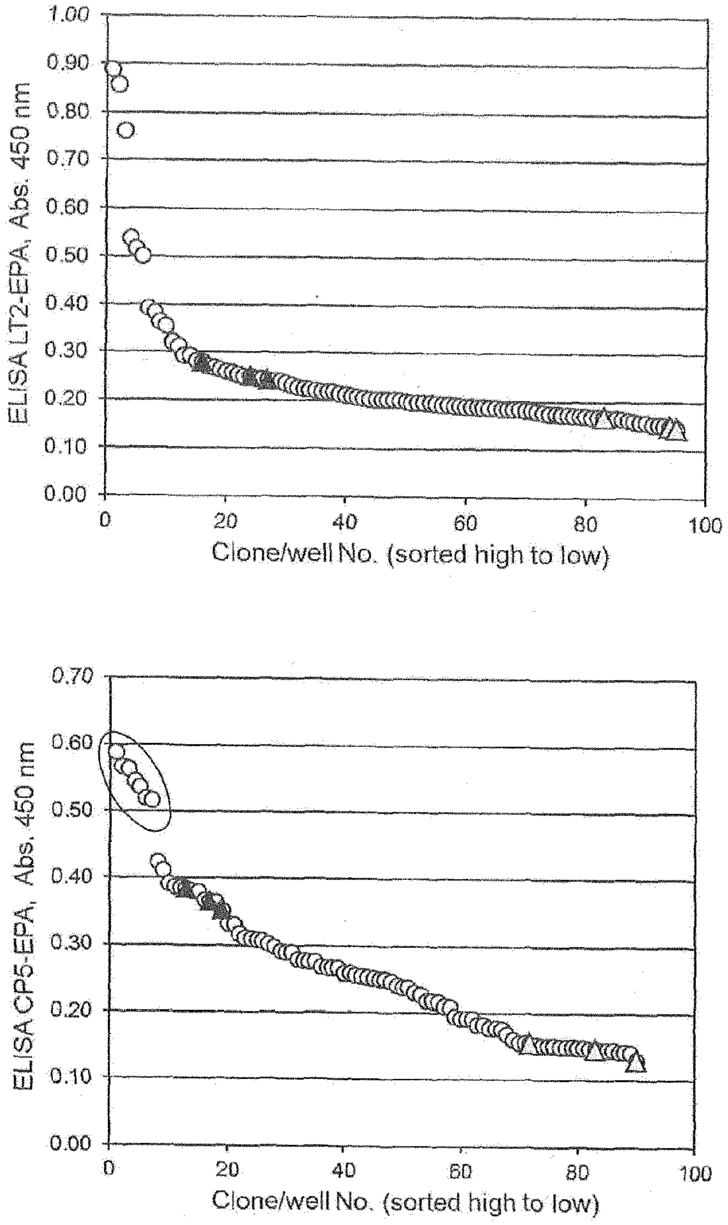

ELISA LT2-EPA, Abs. 450 nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
20
40
60
80
100
Clone/well No. (sorted high to low)
ELISA CP5-EPA, Abs. 450 nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
20
40
60
80
100
Clone/well No. (sorted high to low)

FIG. 3A-B

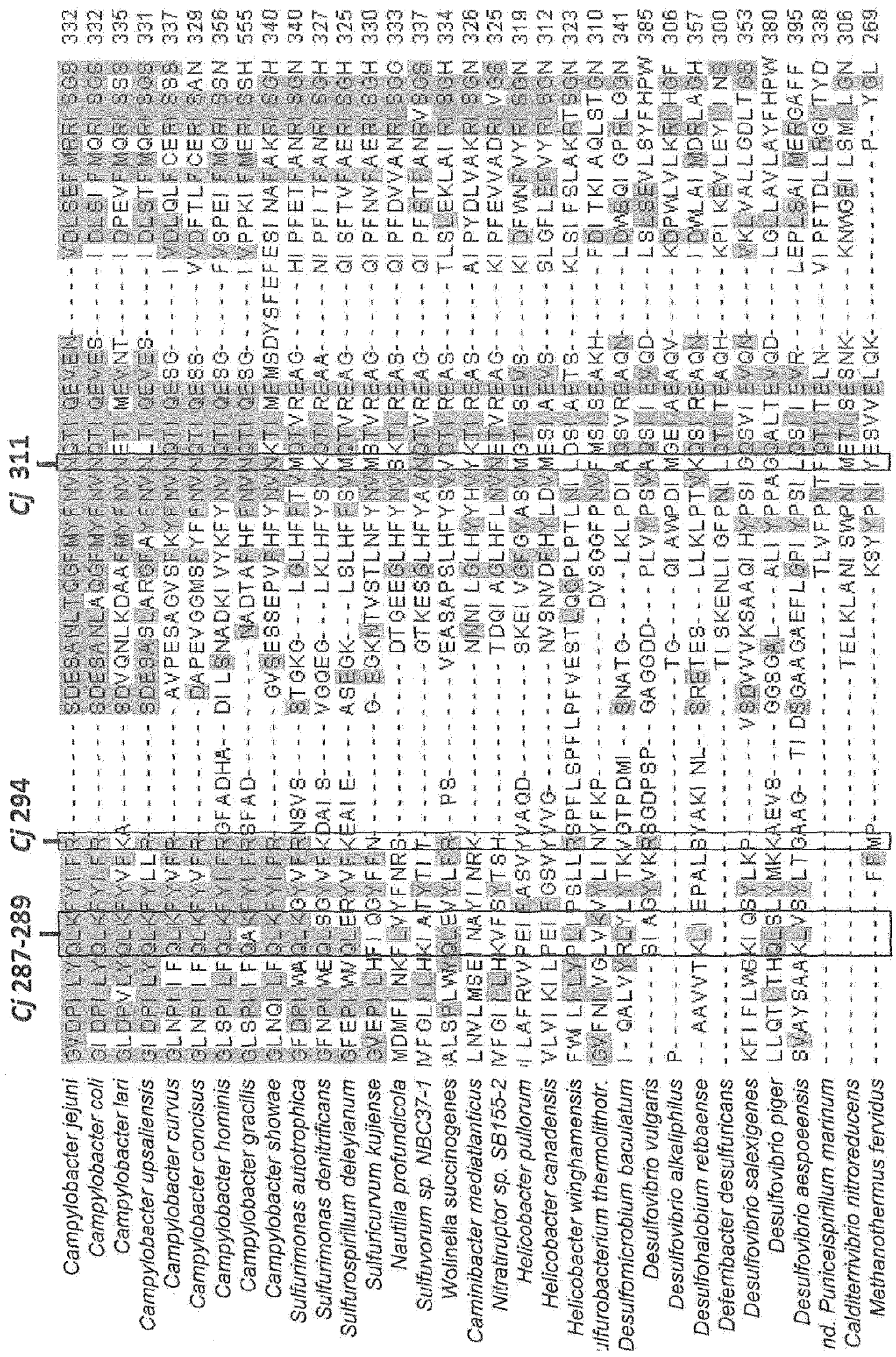

Cj 287-289
Cj 294
Cj 311
Campylobacter jejuni
Campylobacter coli
Campylobacter lari
Campylobacter upsaliensis
Campylobacter curvus
Campylobacter concisus
Campylobacter hominis
Campylobacter gracilis
Campylobacter showae
Sulfurimonas autotrophica
Sulfurimonas denitrificans
Sulfurospirillum deleyianum
Sulfuricurvum kujiense
Nautilia profundicola
Sulfurovum sp. NBC37-1
Wolinella succinogenes
Caminibacter mediatlanticus
Nitratiruptor sp. SB155-2
Helicobacter pullorum
Helicobacter canadensis
Helicobacter winghamensis
ulfurobacterium thermolithotr.
Desulfomicrobium baculatum
Desulfovibrio vulgaris
Desulfovibrio alkaliphilus
Desulfohalobium retbaense
Deferribacter desulfuricans
Desulfovibrio salexigenes
Desulfovibrio piger
Desulfovibrio aespoeensis
nd. Puniceispirillum marinum
Calditerrivibrio nitroreducens
Methanothermus fervidus
332
332
335
331
337
329
356
555
340
340
327
325
330
333
337
334
326
325
319
312
323
310
341
385
306
357
300
353
380
395
338
306
269

FIG. 4A

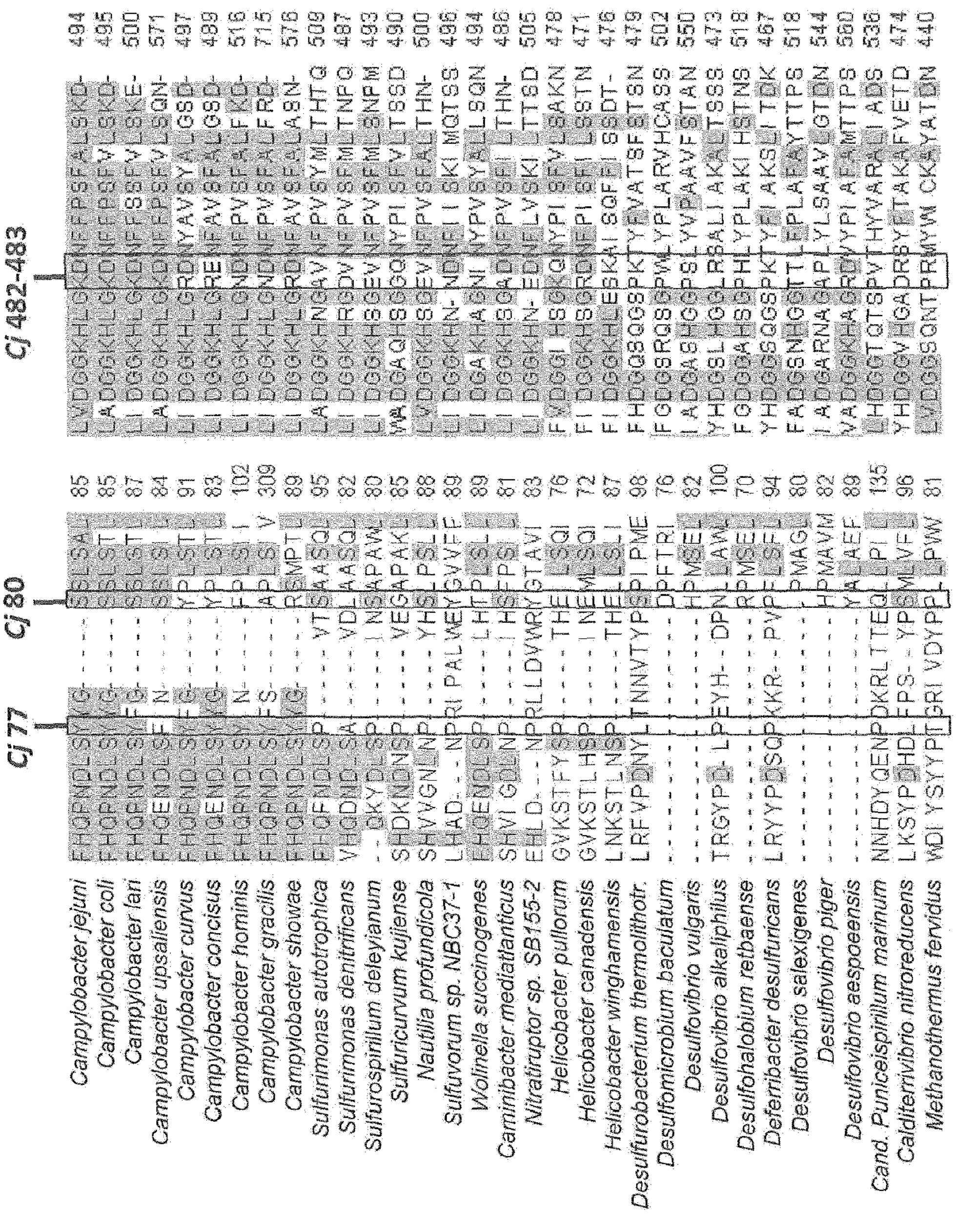

Cj 77
Cj 80
Cj 482-483
Campylobacter jejuni
Campylobacter coli
Campylobacter lari
Campylobacter upsaliensis
Campylobacter curvus
Campylobacter concisus
Campylobacter hominis
Campylobacter gracilis
Campylobacter showae
Sulfurimonas autotrophica
Sulfurimonas denitrificans
Sulfurospirillum deleyianum
Sulfuricurvum kujiense
Nautilia profundicola
Sulfuvorum sp. NBC37-1
Wolinella succinogenes
Caminibacter mediatlanticus
Nitratiruptor sp. SB155-2
Helicobacter pullorum
Helicobacter canadensis
Helicobacter winghamensis
Desulfurobacterium thermolithotr.
Desulfomicrobium baculatum
Desulfovibrio vulgaris
Desulfovibrio alkaliphilus
Desulfohalobium retbaense
Deferribacter desulfuricans
Desulfovibrio salexigenes
Desulfovibrio piger
Desulfovibrio aespoeensis
Cand. Puniceispirillum marinum
Calditerrivibrio nitroreducens
Methanothermus fervidus

FIG. 4B

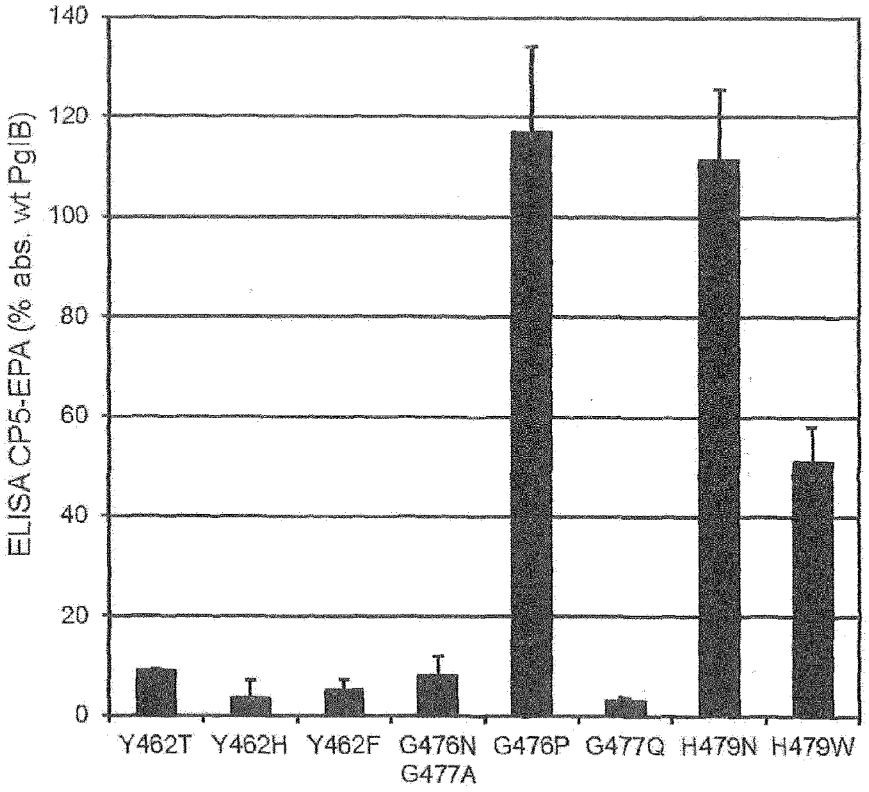

ELISA CP5-EPA (% abs. wt PglB)
140
120
100
80
60
40
20
0
Y462T
Y462H
Y462F
G476N G477A
G476P
G477Q
H479N
H479W

FIG. 5

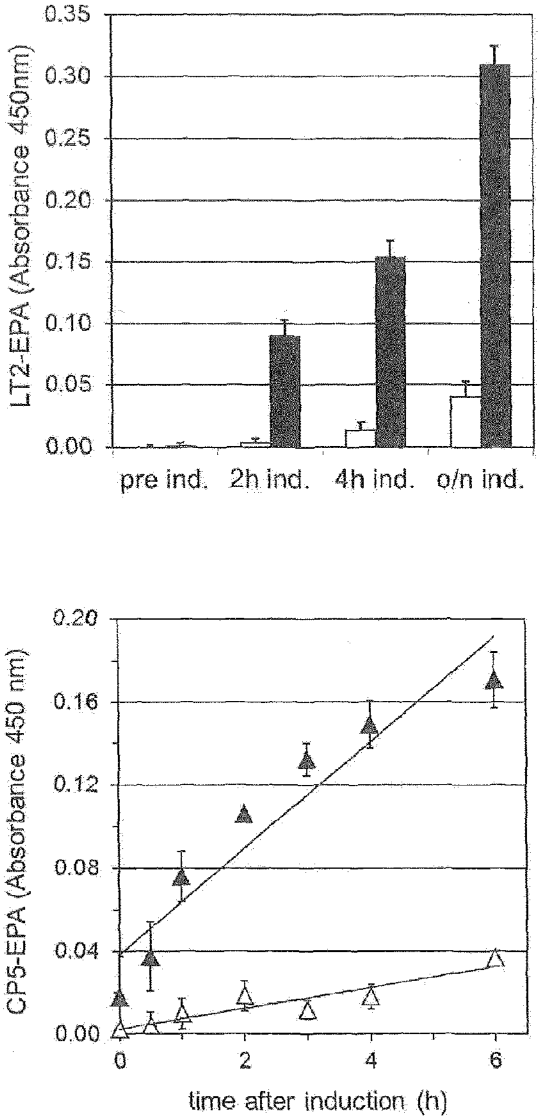

LT2-EPA (Absorbance 450nm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
pre ind.
2h ind.
4h ind.
o/n ind.
CP5-EPA (Absorbance 450 nm)
0.20
0.16
0.12
0.08
0.04
0.00
0
2
4
6
time after induction (h)

FIG. 6A-B

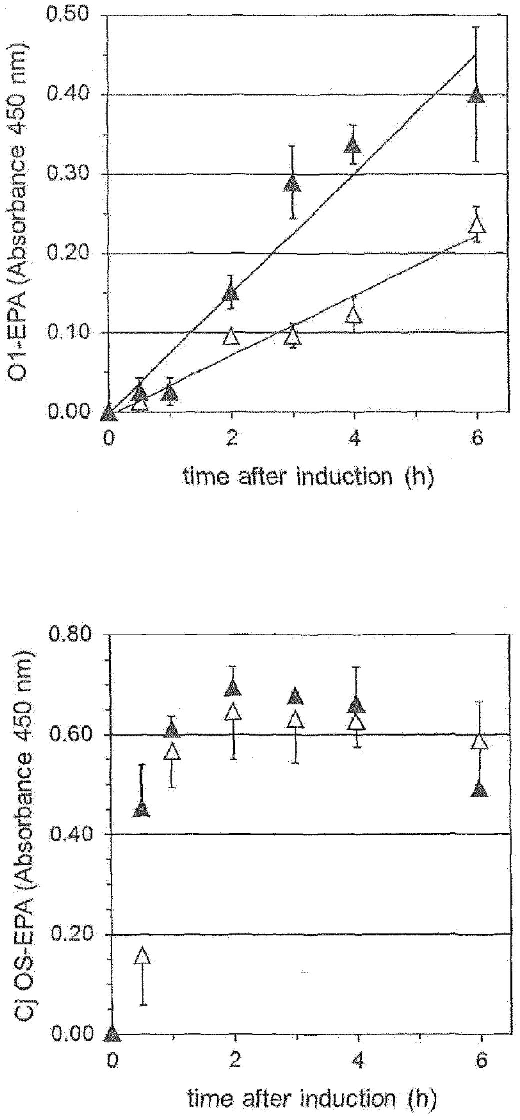

0.50
0.40
0.30
0.20
0.10
0.00
O1-EPA (Absorbance 450 nm)
0
2
4
6
time after induction (h)
0.80
0.60
0.40
0.20
0.00
Cj OS-EPA (Absorbance 450 nm)
0
2
4
6
time after induction (h)

FIG. 6C-D

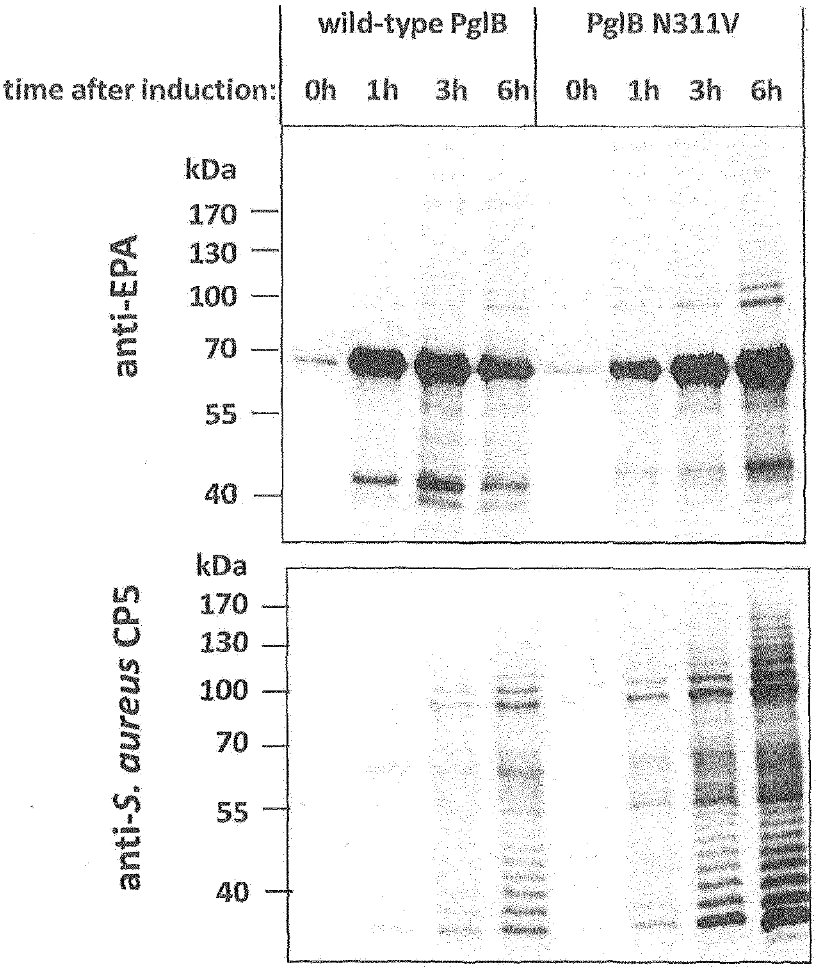

wild-type PglB
PglB N311V
time after induction:
0h
1h
3h
6h
0h
1h
3h
6h
kDa
170
130
100
70
55
40
anti-EPA
kDa
170
130
100
70
55
40
anti-*S. aureus* CP5

FIG. 7A

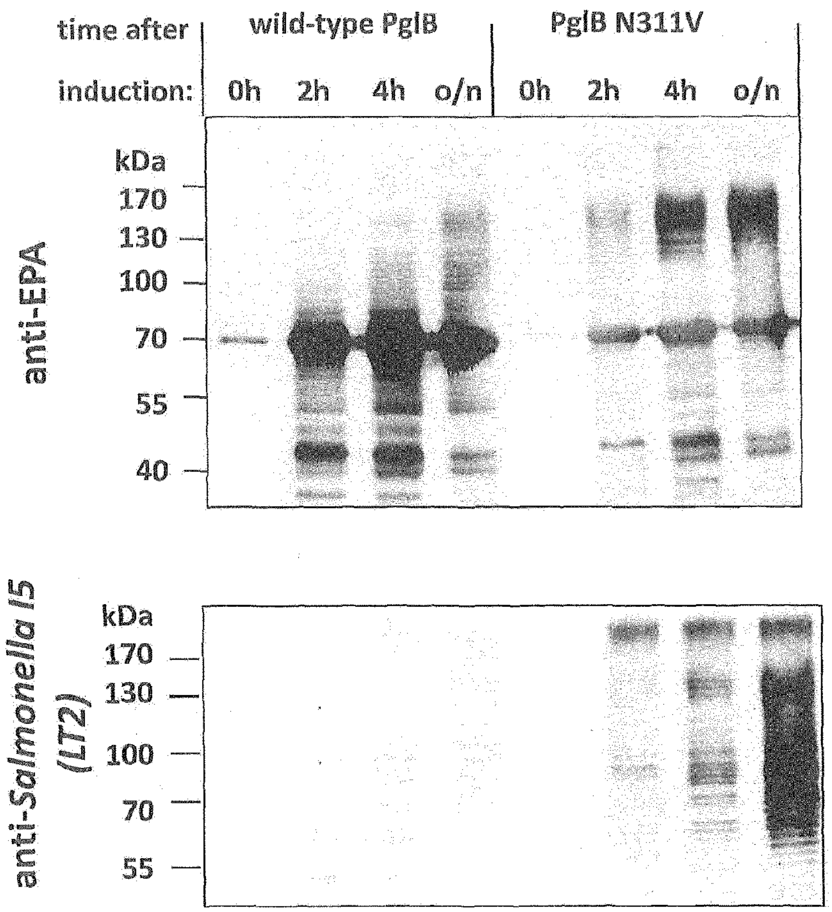

time after
induction:
wild-type PglB
PglB N311V
0h
2h
4h
o/n
0h
2h
4h
o/n
anti-EPA
kDa
170
130
100
70
55
40
anti-*Salmonella I5* (*LT2*)
kDa
170
130
100
70
55

FIG. 7B

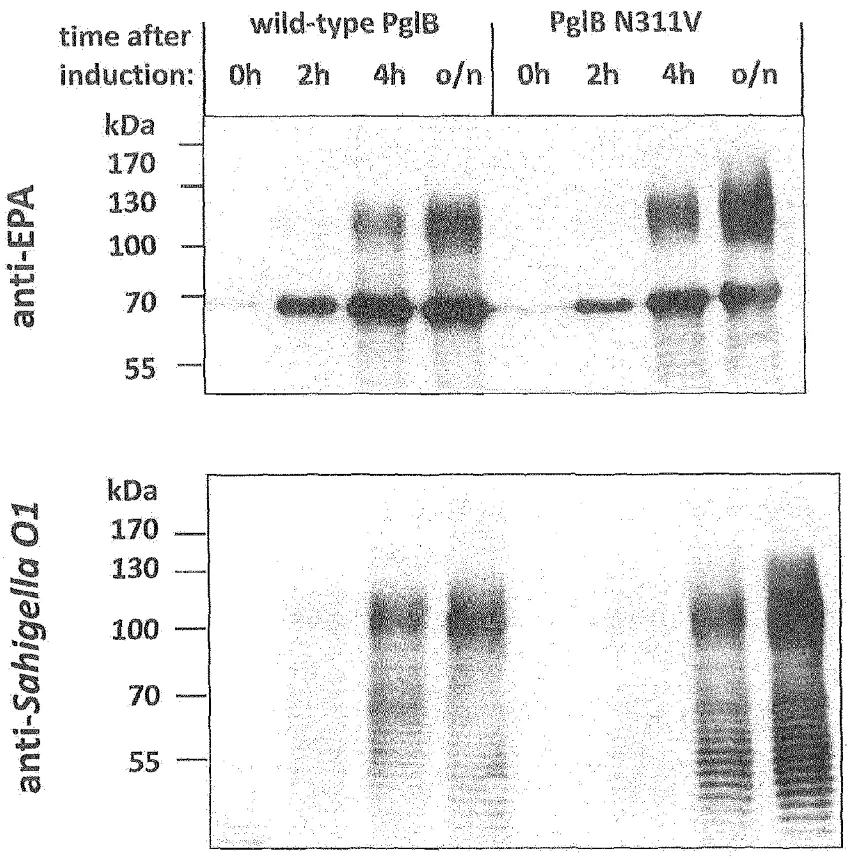

time after induction:
wild-type PglB
PglB N311V
0h
2h
4h
o/n
0h
2h
4h
o/n
kDa
170
130
100
70
55
anti-EPA
kDa
170
130
100
70
55
anti-*Sahigella O1*

FIG. 7C

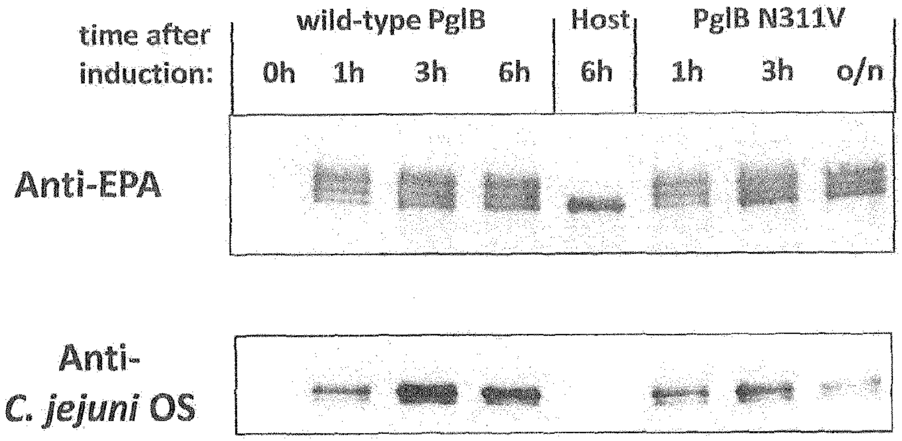
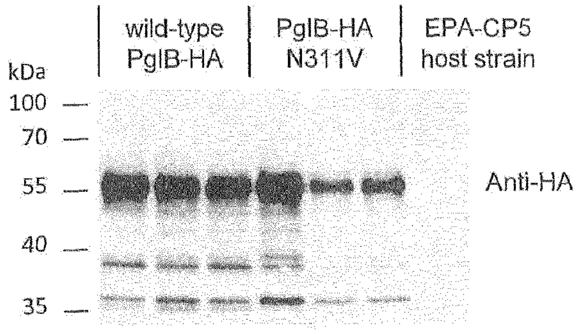
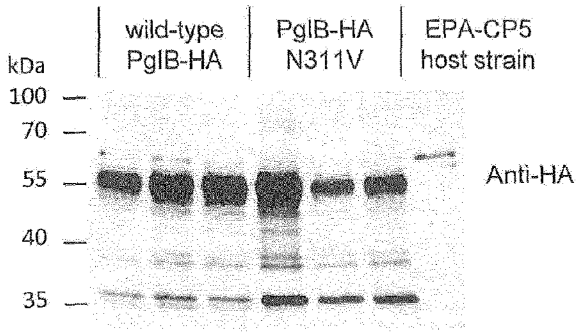

time after
induction:
wild-type PglB
Host
PglB N311V
0h
1h
3h
6h
6h
1h
3h
o/n
Anti-EPA
Anti-
C. jejuni OS

FIG. 7D

Before induction



3h after IPTG and L-arabinose induction



6h after IPTG and L-arabinose induction

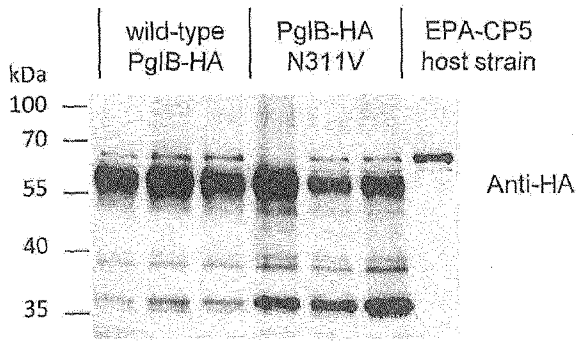


FIG. 8A

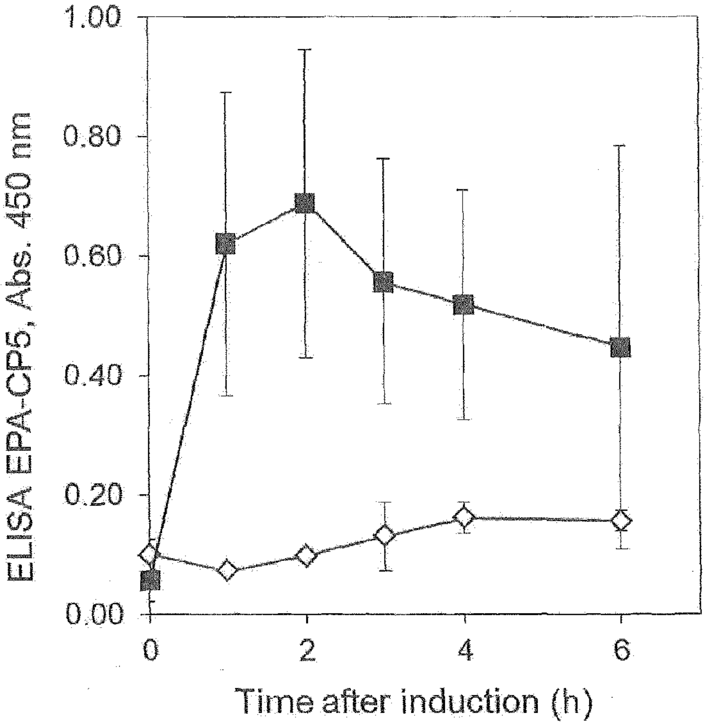

1.00
0.80
0.60
0.40
0.20
0.00
ELISA EPA-CP5, Abs. 450 nm
0
2
4
6
Time after induction (h)

FIG. 8B

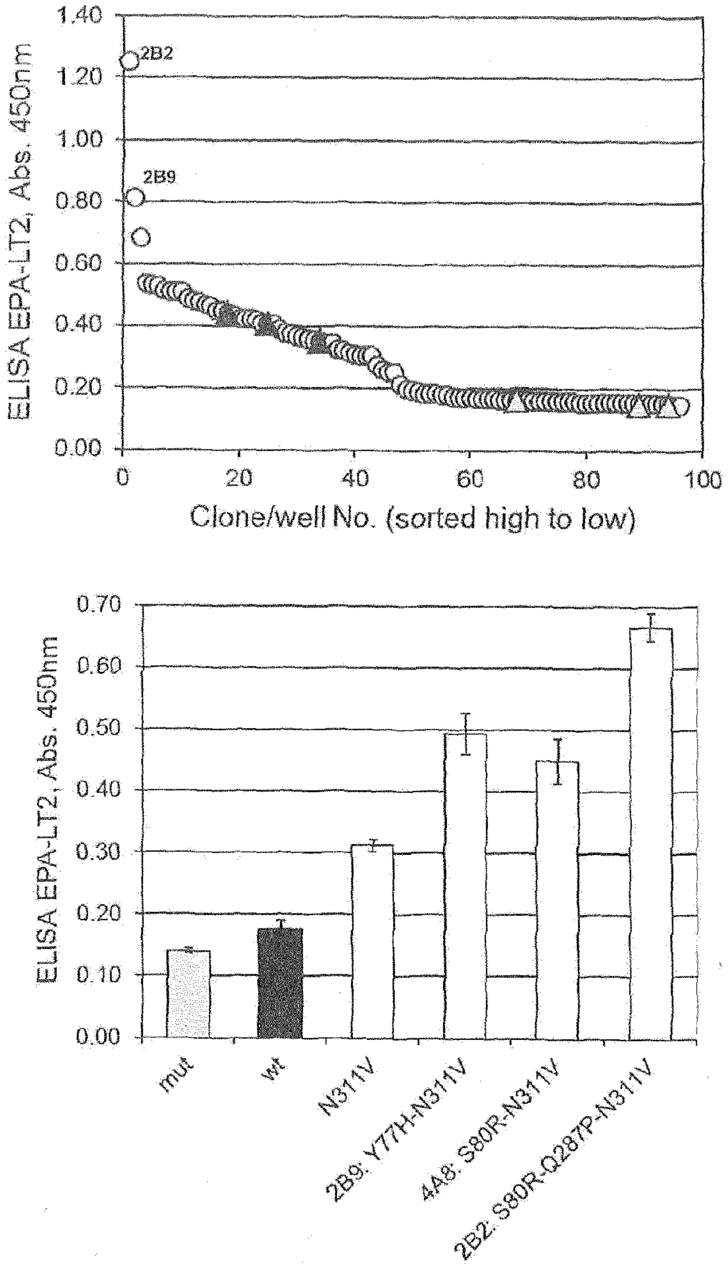

ELISA EPA-LT2, Abs. 450nm
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
2B2
2B9
0
20
40
60
80
100
Clone/well No. (sorted high to low)
ELISA EPA-LT2, Abs. 450nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
mut
wt
N311V
2B9: Y77H-N311V
4A8: S80R-N311V
2B2: S80R-Q287P-N311V

FIG. 9A-B

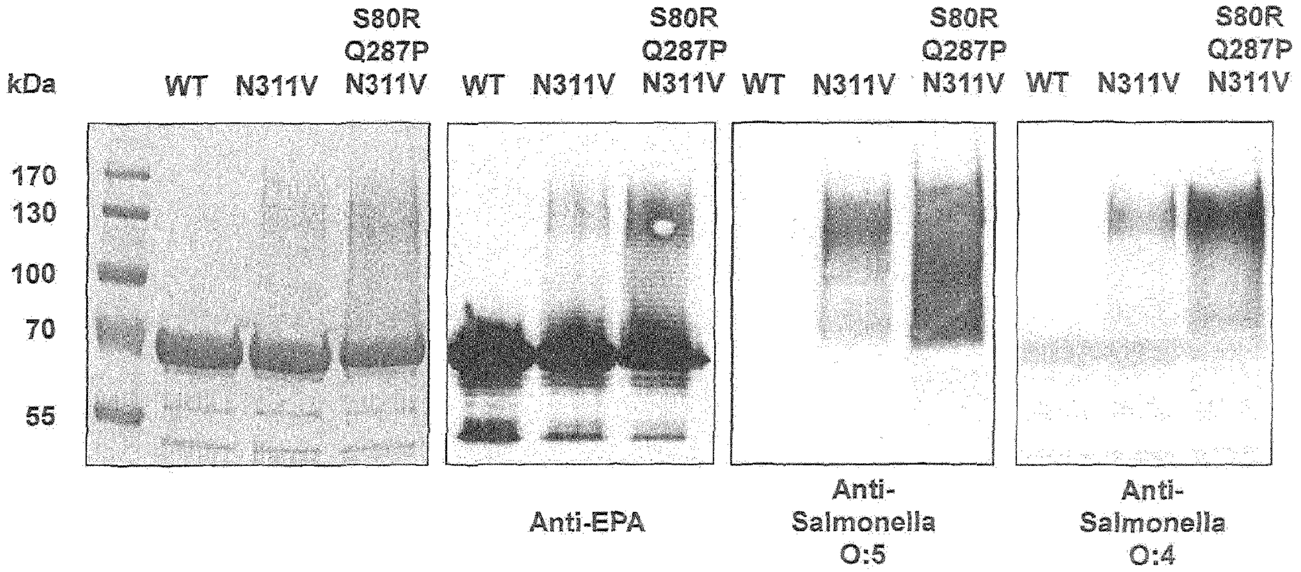

kDa
170
130
100
70
55
WT
N311V
S80R
Q287P
N311V
WT
N311V
S80R
Q287P
N311V
WT
N311V
S80R
Q287P
N311V
WT
N311V
S80R
Q287P
N311V
Anti-EPA
Anti-
Salmonella
O:5
Anti-
Salmonella
O:4

FIG. 9C

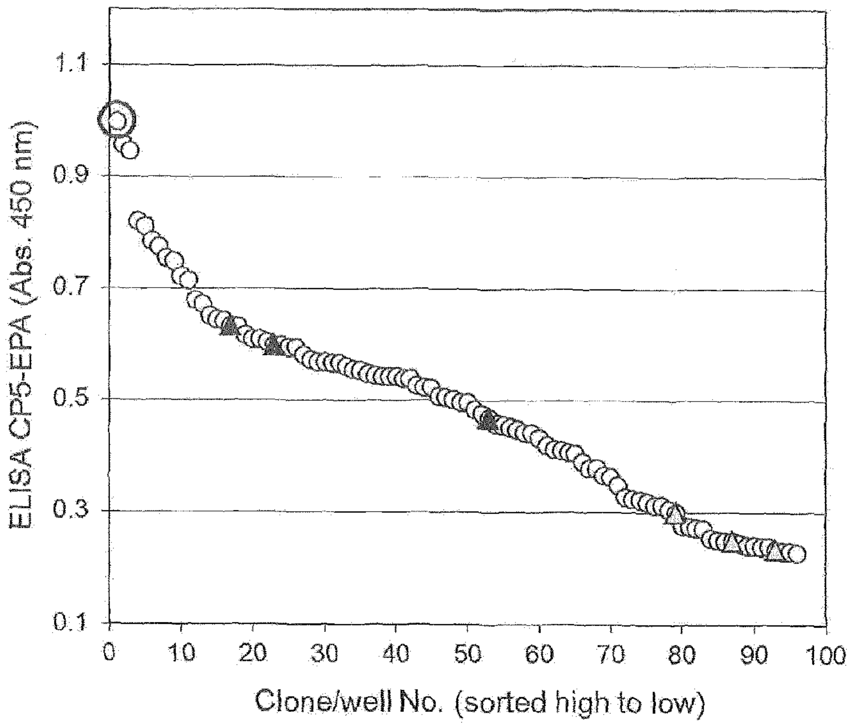

ELISA CP5-EPA (Abs. 450 nm)
1.1
0.9
0.7
0.5
0.3
0.1
0
10
20
30
40
50
60
70
80
90
100
Clone/well No. (sorted high to low)

FIG. 10

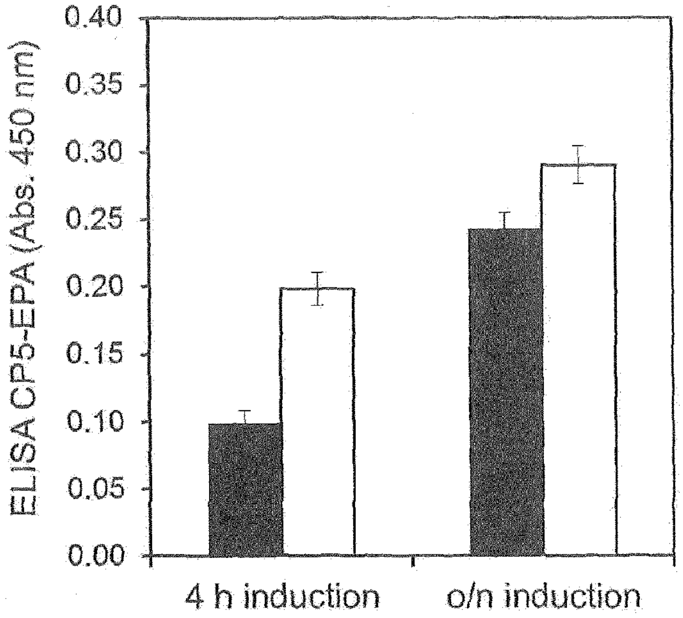

ELISA CP5-EPA (Abs. 450 nm)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
4 h induction
o/n induction

FIG. 11

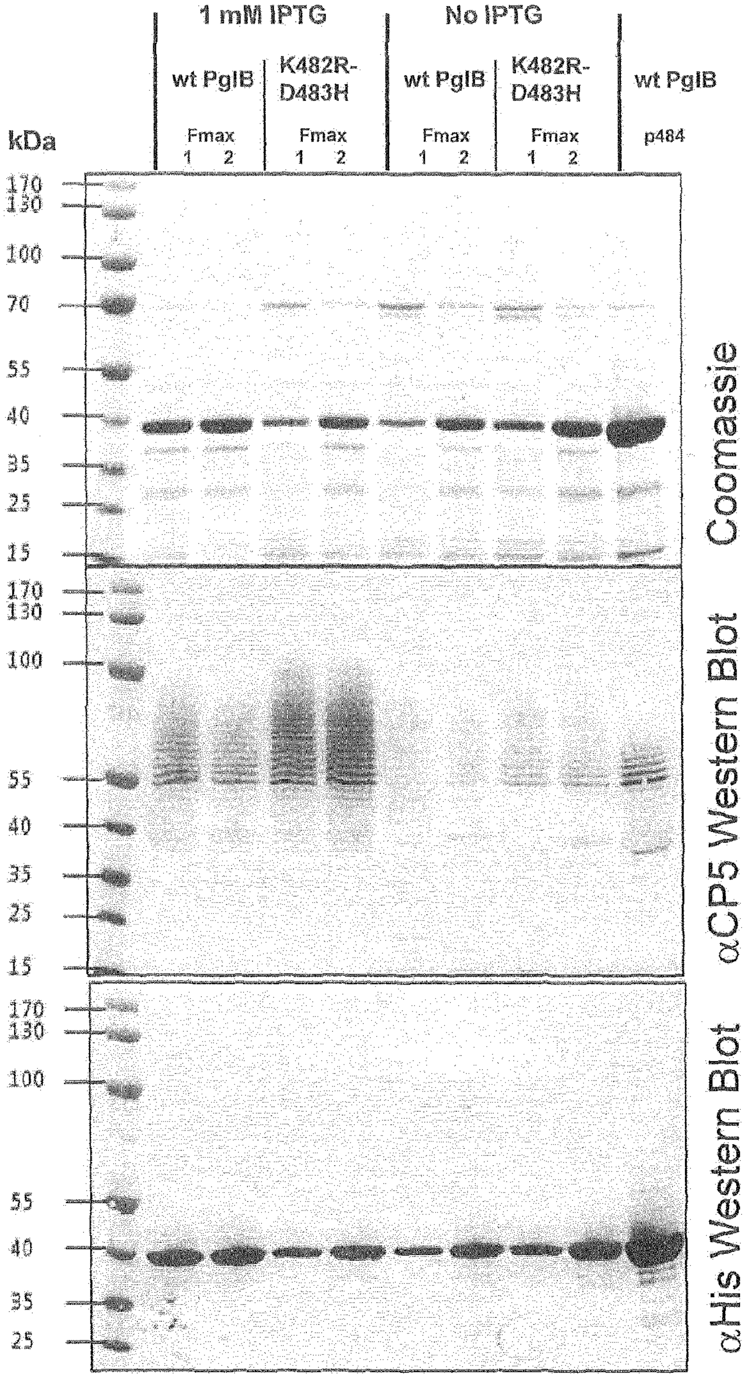

1 mM IPTG
No IPTG
wt PglB
K482R-D483H
wt PglB
K482R-D483H
wt PglB
Fmax 1 2
Fmax 1 2
Fmax 1 2
Fmax 1 2
p484
kDa
170
130
100
70
55
40
35
25
15
Coomassie
170
130
100
55
40
35
25
15
αCP5 Western Blot
170
130
100
55
40
35
25
αHis Western Blot

FIG. 12