CLAIMS

1. A method of preparing a bioconjugate of an *E. coli* O_x-antigen polysaccharide covalently linked to a carrier protein, the method comprising:

(i) providing a recombinant prokaryotic host cell comprising:

a. a nucleotide sequence of an *rfb* gene cluster for the O_x-antigen polysaccharide;

b. a nucleotide sequence encoding the carrier protein comprising at least one glycosylation site comprising a glycosylation consensus sequence Asn-X-Ser(Thr), wherein X is any amino acid except Pro (SEQ ID NO: 1), preferably a glycosylation consensus sequence Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any amino acid except Pro (SEQ ID NO: 2); and

c. a nucleotide sequence encoding an oligosaccharyl transferase $PglB_y$; and

(ii) culturing the recombinant prokaryotic host cell under conditions for production of the bioconjugate,

wherein:

when the O_x- antigen is O1A antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of N311V, K482R, D483H, and A669V;

when the O_x-antigen is glucosylated O4 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutation N311V or the amino acid mutations Y77H and N311V, and the recombinant host cell further comprises a sequence encoding a glucosyltransferase GtrS having at least 80% identity to SEQ ID NO: 4 and being capable of modifying an *E. coli* O4 antigen polysaccharide by addition of glucose to produce the *E. coli* glucosylated O4 antigen polysaccharide, and nucleotide sequences encoding a translocase GtrA and a glycosyltransferase GtrB having at least 80% sequence identity to SEQ ID NOs: 7 and 8 respectively, wherein the translocase is capable of translocating

bactoprenol linked glucose and the glycosyltransferase is capable of glucosylating bactoprenol;

when the O_x-antigen is O8 antigen polysaccharide, the $PglB_y$ comprises no amino acid mutations at positions 77, 80, 287, 289, 311, 482, 483 and 669;

when the O_x-antigen is O15 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of N311V, K482R, D483H, and A669V;

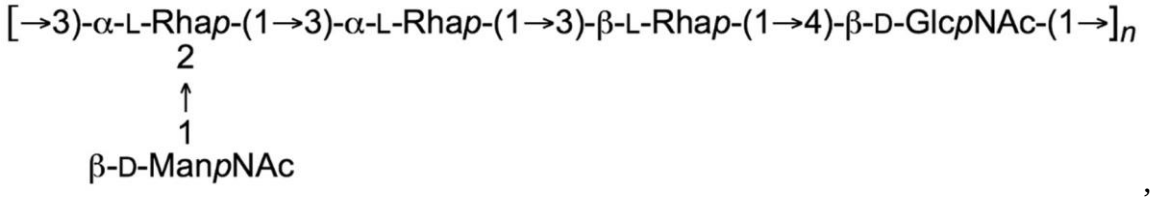
when the O_x-antigen is O16 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of Y77H, S80R, Q287P, K289R, and N311V;

when the O_x-antigen is O18A antigen polysaccharide, the $PglB_y$ comprises no amino acid mutations at positions 77, 80, 287, 289, 311, 482, 483 and 669; and

when the O_x-antigen is O75 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutation of N311V,

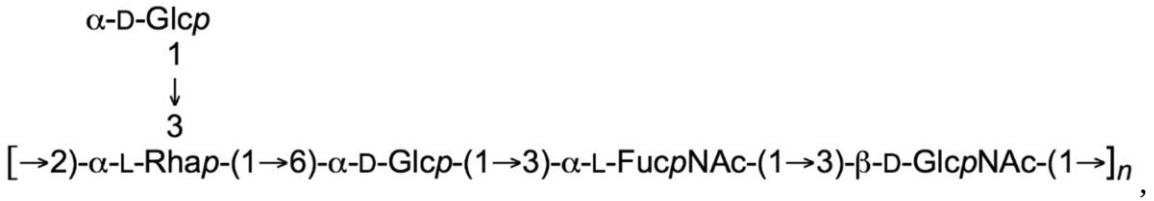
wherein in each case the amino acid mutations are relative to the wild-type PglB having the amino acid sequence of SEQ ID NO: 6, and

wherein the O1A, glucosylated O4, O8, O15, O16, O18A, and O75 antigen polysaccharides have the structures of Formulas (O1A):



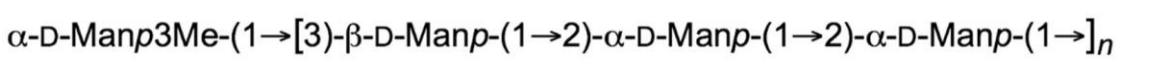
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(O4-Glc+):



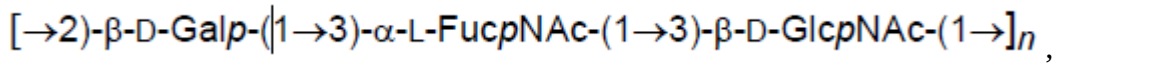
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(O8):



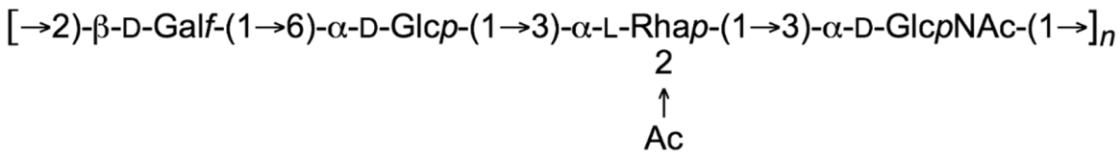
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(O15):



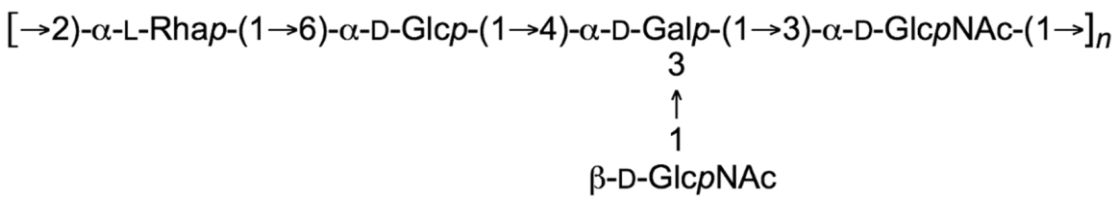
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(O16):



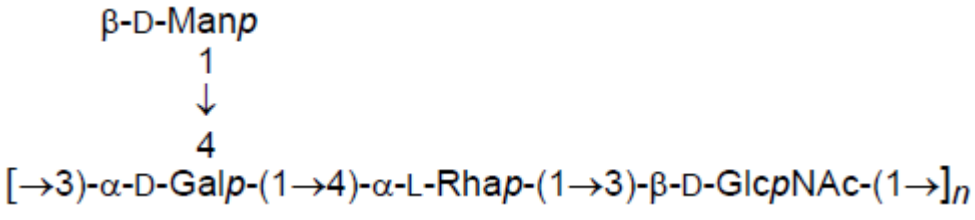
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(O18A):



,

and (O75):



, respectively, and each n is independently an integer of 1 to 100, preferably 3 to 50, e.g. 5 to 40, e.g. 7 to 25, e.g. 10 to 20.

2. A method of preparing a bioconjugate of an *E. coli* O_x-antigen polysaccharide covalently linked to a carrier protein, the method comprising:

(i) providing a recombinant prokaryotic host cell comprising:

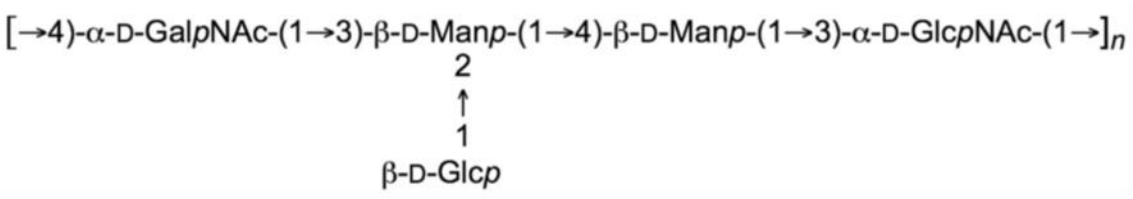
a. a nucleotide sequence of an *rfb* gene cluster for the O_x-antigen polysaccharide;

b. a nucleotide sequence encoding the carrier protein comprising at least one glycosylation site comprising a glycosylation consensus sequence Asn-X-Ser(Thr), wherein X is any amino acid except Pro (SEQ ID NO: 1), preferably a glycosylation consensus sequence Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any amino acid except Pro (SEQ ID NO: 2); and

c. a nucleotide sequence encoding an oligosaccharyl transferase $PglB_y$; and

(ii) culturing the recombinant prokaryotic host cell under conditions for production of the bioconjugate, wherein:

the O_x-antigen is O6A antigen polysaccharide, the PglBy comprises the amino acid mutations of N311V, K482R, D483H, and A669V, wherein the amino acid mutations are relative to the wild-type PglB having the amino acid sequence of SEQ ID NO: 6; and the O6A antigen polysaccharide has the following formulae:



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wherein each n is independently an integer of 1 to 100, preferably 3 to 50, e.g. 5 to 40, e.g. 7 to 25, e.g. 10 to 20.

3. The method of claim 1, wherein the O_x-antigen is glucosylated O4 antigen polysaccharide, and the $PglB_y$ comprises the amino acid mutation N311V or the amino acid mutations Y77H and N311V relative to wild-type PglB having the amino acid sequence of SEQ ID NO: 6.

4. The method of claim 3, wherein the recombinant prokaryotic host cell further comprises a sequence encoding a GtrS having the amino acid sequence of SEQ ID NO: 4, and nucleotide sequences encoding a GtrA and a GtrB having the amino acid sequences of SEQ ID NOs: 7 and 8, respectively.

5. A method of preparing a bioconjugate of an *E. coli* O_x-antigen polysaccharide covalently linked to a carrier protein, the method comprising:

(i) providing a recombinant prokaryotic host cell comprising:

a. a nucleotide sequence of an *rfb* gene cluster for the O_x-antigen polysaccharide;

b. a nucleotide sequence encoding the carrier protein comprising at least one glycosylation site comprising a glycosylation consensus sequence Asn-X-Ser(Thr), wherein X is any amino acid except Pro (SEQ ID NO: 1), preferably a glycosylation consensus sequence Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any amino acid except Pro (SEQ ID NO: 2); and

c. a nucleotide sequence encoding an oligosaccharyl transferase $PglB_y$; and

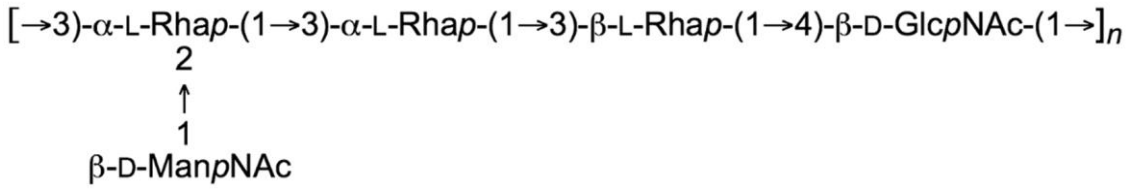
(ii) culturing the recombinant prokaryotic host cell under conditions for production of the bioconjugate,

wherein the $PglB_y$ comprises the amino acid mutation N311V relative to the wild-type PglB having the amino acid sequence of SEQ ID NO: 6,

wherein the O_x- antigen is O1A antigen polysaccharide, glucosylated O4 antigen polysaccharide, O6A antigen polysaccharide, O15 antigen polysaccharide, O16 antigen polysaccharide, or O75 antigen polysaccharide,

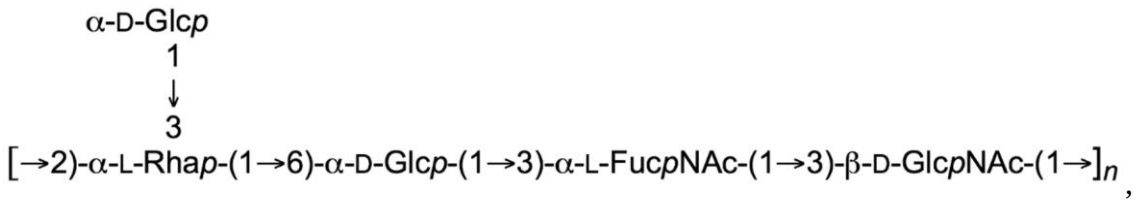
and when the O_x-antigen is glucosylated O4 antigen polysaccharide, the recombinant prokaryotic host cell further comprises a sequence encoding a glucosyltransferase GtrS having at least 80% identity to SEQ ID NO: 4 and being capable of modifying an *E. coli* O4 antigen polysaccharide by addition of glucose to produce the *E. coli* glucosylated O4 antigen polysaccharide, and nucleotide sequences encoding a translocase GtrA and a glycosyltransferase GtrB having at least 80% sequence identity to SEQ ID NOs: 7 and 8, respectively, wherein the translocase is capable of translocating bactoprenol linked glucose and the glycosyltransferase is capable of glucosylating bactoprenol, and

wherein the O1A, glucosylated O4, O6A, O15, O16, and O75 antigen polysaccharides have the structures of Formulas (O1A):

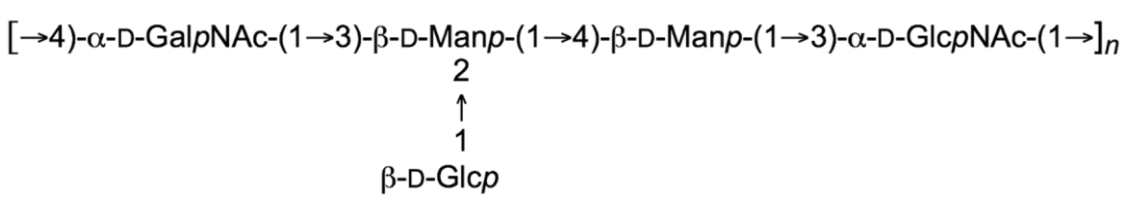


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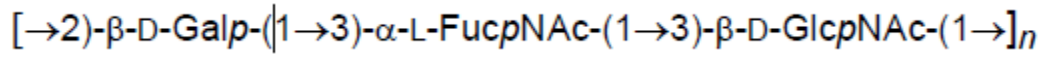
(O4-Glc+):



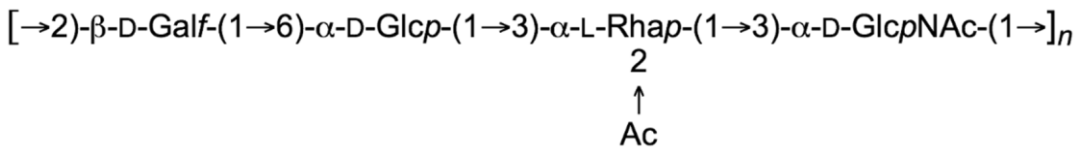
(O6A):



(O15):

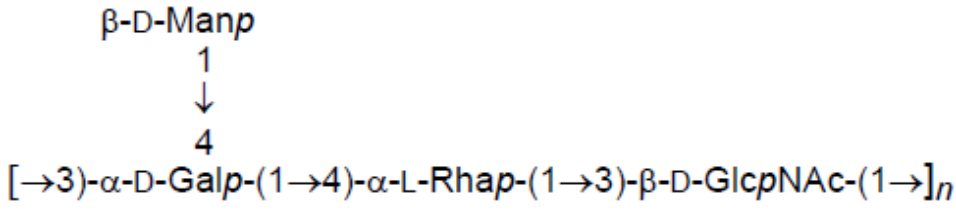


(O16):



, and

(O75):



, respectively, and each n is independently an integer of 1 to 100, preferably 3 to 50, e.g. 5 to 40, e.g. 7 to 25, e.g. 10 to 20.

6. The method of any one of claims 1 to 5, further comprising isolating the bioconjugate from the recombinant prokaryotic host cell.

7. The method of any one of claims 1 to 6, wherein the carrier protein is selected from the group consisting of detoxified Exotoxin A of *P. aeruginosa* (EPA), *E. coli* flagellin (FliC), CRM197, maltose binding protein (MBP), Diphtheria toxoid, Tetanus toxoid,

detoxified hemolysin A of *S. aureus*, clumping factor A, clumping factor B, *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, *Streptococcus pneumoniae* Pneumolysin, Keyhole limpet hemocyanin (KLH), *P. aeruginosa* PcrV, outer membrane protein of *Neisseria meningitidis* (OMPC), and protein D from non-typeable *Haemophilus influenzae*.

8. The method of any one of claims 1-6, wherein the carrier protein is detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA), preferably wherein the EPA carrier protein comprises 1-10, preferably 2-4, more preferably 4, of the glycosylation sites, preferably wherein each glycosylation site comprises a glycosylation consensus sequence having SEQ ID NO: 2, preferably wherein the EPA carrier protein comprises SEQ ID NO: 3.

9. The method of any one of claims 1 to 6, wherein the carrier protein is detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA), wherein the EPA carrier protein comprises 2-4 glycosylation sites, wherein each glycosylation site comprises a glycosylation consensus sequence having SEQ ID NO: 2.

10. The method of any one of claims 1-9, wherein the recombinant prokaryotic host cell is an *E. coli* cell, e.g. an E. coli K-12 strain, such as strain W3110.

11. A recombinant prokaryotic host cell for preparing a bioconjugate of an *E. coli* O_x-antigen polysaccharide covalently linked to a carrier protein, the recombinant prokaryotic host cell comprising:

a. a nucleotide sequence of an *rfb* gene cluster for the O_x-antigen polysaccharide;

b. a nucleotide sequence encoding the carrier protein comprising at least one glycosylation site comprising a glycosylation consensus sequence Asn-X-Ser(Thr), wherein X is any amino acid except Pro (SEQ ID NO: 1),

preferably a glycosylation consensus sequence Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any amino acid except Pro (SEQ ID NO: 2); and

c. a nucleotide sequence encoding an oligosaccharyl transferase $PglB_y$,

wherein:

when the O_x- antigen is O1A antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of N311V, K482R, D483H, and A669V;

when the O_x-antigen is glucosylated O4 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutation N311V or the amino acid mutations Y77H and N311V, and the recombinant host cell further comprises a sequence encoding a glucosyltransferase GtrS having at least 80% identity to SEQ ID NO: 4 and being capable of modifying an *E. coli* O4 antigen polysaccharide by addition of glucose to produce the *E. coli* glucosylated O4 antigen polysaccharide, and nucleotide sequences encoding a translocase GtrA and a glycosyltransferase GtrB having at least 80% sequence identity to SEQ ID NOs: 7 and 8 respectively, wherein the translocase is capable of translocating bactoprenol linked glucose and the glycosyltransferase is capable of glucosylating bactoprenol;

when the O_x-antigen is O8 antigen polysaccharide, the $PglB_y$ comprises no amino acid mutations at positions 77, 80, 287, 289, 311, 482, 483 and 669;

when the O_x-antigen is O15 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of N311V, K482R, D483H, and A669V;

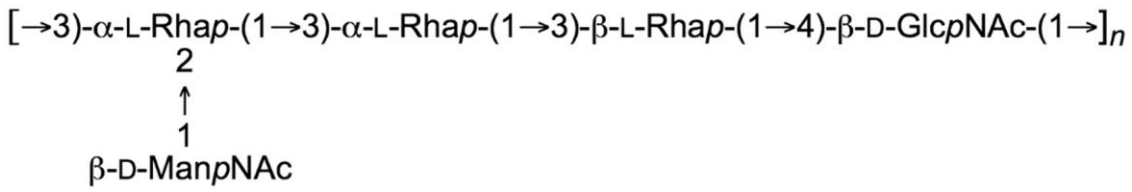
when the O_x-antigen is O16 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutations of Y77H, S80R, Q287P, K289R, and N311V;

when the O_x-antigen is O18A antigen polysaccharide, the $PglB_y$ comprises no amino acid mutations at positions 77, 80, 287, 289, 311, 482, 483 and 669; and

when the O_x-antigen is O75 antigen polysaccharide, the $PglB_y$ comprises the amino acid mutation of N311V,

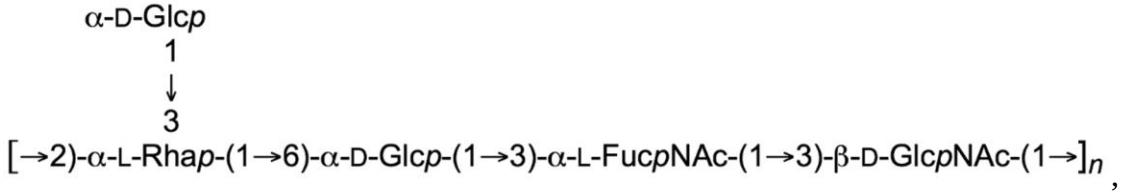
wherein in each case the amino acid mutations are relative to the wild-type PglB having the amino acid sequence of SEQ ID NO: 6, and

wherein the O1A, glucosylated O4, O8, O15, O16, O18A, and O75 antigen polysaccharides have the structures of Formulas (O1A):



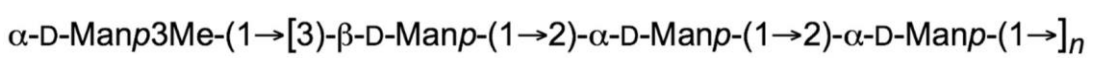
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(O4-Glc+):



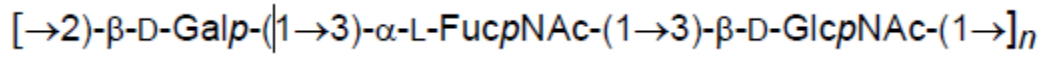
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(O8):



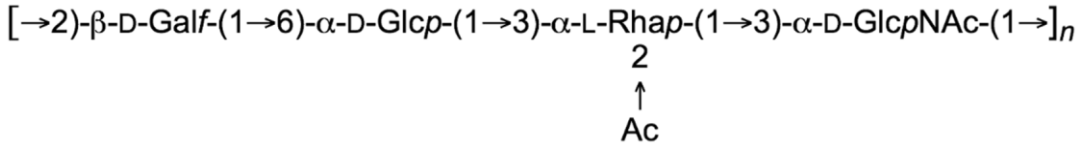
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(O15):



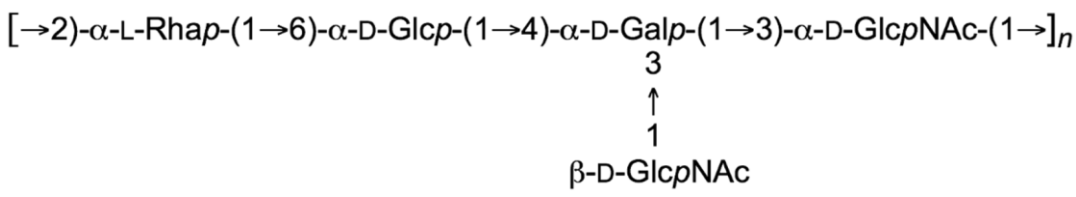
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(O16):



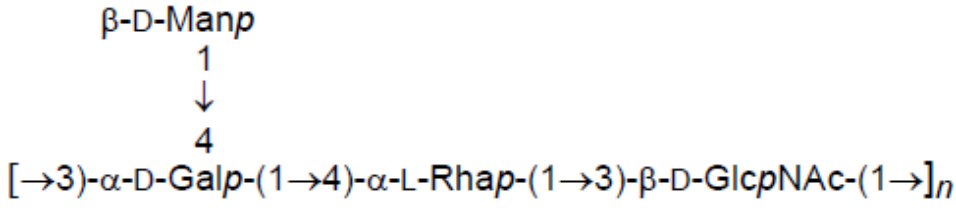
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(O18A):



, and

(O75):



, respectively, and each n is independently an integer of 1 to 100, preferably 3 to 50, e.g. 5 to 40, e.g. 7 to 25, e.g. 10 to 20.

12. The recombinant prokaryotic host cell of claim 11, wherein the O_x-antigen is glucosylated O4 antigen polysaccharide, and the $PglB_y$ comprises the amino acid mutation N311V or the amino acid mutations Y77H and N311V relative to wild-type PglB having the amino acid sequence of SEQ ID NO: 6.

13. The recombinant prokaryotic host cell of claim 12, wherein the recombinant prokaryotic host cell further comprises a sequence encoding a GtrS having the amino acid sequence of SEQ ID NO: 4, and nucleotide sequences encoding a GtrA and a GtrB having the amino acid sequences of SEQ ID NOs: 7 and 8, respectively.

14. The recombinant prokaryotic host cell of any one of claims 11 to 13, wherein the carrier protein is selected from the group consisting of detoxified Exotoxin A of *P. aeruginosa* (EPA), *E. coli* flagellin (FliC), CRM197, maltose binding protein (MBP), Diphtheria toxoid, Tetanus toxoid, detoxified hemolysin A of *S. aureus*, clumping factor A, clumping factor B, *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, *Streptococcus pneumoniae* Pneumolysin, Keyhole limpet hemocyanin (KLH), *P. aeruginosa* PcrV, outer membrane protein of *Neisseria meningitidis* (OMPC), and protein D from non-typeable *Haemophilus influenzae.*

15. The recombinant prokaryotic host cell of any one of claims 11-13, wherein the carrier protein is detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA), preferably wherein the EPA carrier protein comprises 1-10, preferably 2-4, more preferably 4, of the

glycosylation sites, preferably wherein each glycosylation site comprises a glycosylation consensus sequence having SEQ ID NO: 2, preferably wherein the EPA carrier protein comprises SEQ ID NO: 3.

16. The recombinant prokaryotic host cell of any one of claims 11 to 13, wherein the carrier protein is detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA), wherein the EPA carrier protein comprises 2-4 glycosylation sites, wherein each glycosylation site comprises a glycosylation consensus sequence having SEQ ID NO: 2.