OUTER MEMBRANE VESICLE VACCINES FOR THE PREVENTION OF N. GONORRHOEAE AND METHODS OF MAKING AND USING THEREOF

GOVERNMENT LICENSE RIGHTS

[0001] This invention was made with U.S. Government support under Agreement Number 75A50122C00028, awarded by the U.S. Department of Health and Human Services. The U.S. Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Patent Application No. 63/568,920, filed March 22, 2024, and U.S. Provisional Patent Application No. 63/663,894, filed June 25, 2024, the contents of each of which are incorporated by reference herein in their entireties.

SEQUENCE LISTING

[0003] This application contains an electronic Sequence Listing which has been submitted in XML file format with this application, the entire content of which is incorporated by reference herein in its entirety. The Sequence Listing XML file submitted with this application is entitled "14197-021-228_SEQLISTING.xml", was created on March 18, 2025, and is 118,285 bytes in size.

1. FIELD

[0004] The present disclosure provides novel outer membrane vesicles (OMVs) comprising selective bacterial antigens (*e.g.*, glycan antigens and/or protein antigens), use of such OMVs as vaccines (*e.g.*, vaccines against *Neisseria gonorrhoeae*) to protect against infectious diseases, and methods of making the same. The present disclosure also provides genetically engineered *Escherichia coli* for producing the OMVs and methods of making the same.

2. BACKGROUND

[0005] *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is an urgent threat for antimicrobial resistance (AMR), with resistance documented in almost all antimicrobial treatments. Effective gonococcal vaccine is urgently needed to mitigate AMR risks and to protect vulnerable populations. However, there is currently no licensed vaccine against *N. gonorrhoeae*.

[0006] Vaccine approaches based on OMVs derived from *N. gonorrhoeae* have not been successful. One reason is that *N. gonorrhoeae* infections do not elicit protective immunity. As such, OMVs derived from *N. gonorrhoeae,* which resemble *N. gonorrhoeae* closely, bear the same risk as *N. gonorrhoeae* of not eliciting sufficient protective immunity. In particular, the array of phase- and antigenically variable proteins present on the surface of *N. gonorrhoeae* may elicit non-protective antibody responses, which contribute to the immune evasion and suppression mechanisms of *N. gonorrhoeae*. For example, gonococcal lipooligosaccharide (LOS) can bind and inactivate complement cascade components and prevent the formation of membrane-attack complex. In addition, the immune suppression mechanisms of *N. gonorrhoeae* can block the production of proinflammatory cytokines and inhibit protective Th1 and Th2 responses.

[0007] Similarly, vaccine approaches using heat-killed bacteria and pilus protein also fail to elicit protective immunity because these approaches induce immune suppression mechanisms utilized by *N. gonorrhoeae*.

[0008] Thus, there remains a need for effective gonococcal vaccines that have low cost, simple production process, and high immunogenicity, but without any of the immune evasion and suppression exhibited by *N. gonorrhoeae*.

3. SUMMARY

[0009] In one aspect, the present disclosure provides an *Escherichia coli* (*E. coli*)-derived outer membrane vesicle (OMV) comprising a glycan antigen and a protein antigen of *N. gonorrhoeae*. In certain embodiments, the OMV further comprises a modified lipid A.

[0010] In one aspect, the present disclosure provides an *Escherichia coli* (*E. coli*)-derived outer membrane vesicle (OMV) comprising a glycan antigen and a protein antigen of a pathogen, and a modified lipid A. In certain embodiments, the pathogen is *N. gonorrhoeae*.

[0011] In certain embodiments, the modified lipid A is produced by at least one lipid A modification enzyme selected from the group consisting of msbB, pagL, lpxE, pagP, and combinations thereof. In certain embodiments, the msbB, pagL, and/or lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV. In certain embodiments, the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes. In certain embodiments, the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

[0012] In certain embodiments, the modified lipid A is produced by at least one lipid A modification enzyme selected from the group consisting of pagL, lpxE, pagP, and combinations

thereof. In certain embodiments, the pagL and/or lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV. In certain embodiments, the *E. coli* comprises genetic disruption of *eptA* and/or *lpxT* genes. In certain embodiments, the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

[0013] In certain embodiments, the modified lipid A comprises a mixture of heterogeneous lipid A species having different chemical structures.

[0014] In certain embodiments, the modified lipid A comprises at least one lipid A species comprising only one phosphate group. In certain embodiments, the modified lipid A comprises at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone. In certain embodiments, at least 60% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone. In certain embodiments, between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone.

[0015] In certain embodiments, the modified lipid A comprises at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone. In certain embodiments, at least 60% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone. In certain embodiments, between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone.

[0016] In certain embodiments, the modified lipid A comprises at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A. In certain embodiments, at least 50% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A. In certain embodiments, between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A.

[0017] In certain embodiments, the modified lipid A comprises at least one lipid A species comprising at least three C14-3OH acyl chains. In certain embodiments, at least 60% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains. In certain embodiments, between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains.

[0018] In certain embodiments, the modified lipid A comprises at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone. In certain embodiments, at least 30% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone. In certain embodiments, between 40% and 50% of the amount of the modified lipid A

comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone.

[0019] In certain embodiments, the modified lipid A comprises at least one lipid A species comprising a C12 acyl chain. In certain embodiments, at least 60% of the amount of the modified lipid A comprises a C12 acyl chain. In certain embodiments, between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

[0020] In certain embodiments, the protein antigen and glycan antigen are associated with the outer membrane of the OMV.

[0021] In certain embodiments, the protein antigen comprises a MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 9. In certain embodiments, the MsrA/B polypeptide comprises the amino acid sequence of SEQ ID NO: 9. In certain embodiments, the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14.

[0022] In certain embodiments, the protein antigen comprises a MetQ polypeptide. In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7.

[0023] In certain embodiments, the protein antigen comprises an AniA polypeptide. In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4. In certain embodiments, the AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

[0024] In certain embodiments, the protein antigen comprises a NHBA polypeptide. In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, the NHBA-MetQ

fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

[0025] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising an AniA polypeptide that is associated with the outer membrane of the OMV, optionally the OMV comprises a glycan antigen. In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4. In certain embodiments, the AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

[0026] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a MetQ polypeptide and a glycan antigen of a pathogen, wherein the MetQ polypeptide and the glycan antigen are associated with the outer membrane of the OMV. In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7.

[0027] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a NHBA polypeptide and a glycan antigen of a pathogen, wherein the NHBA polypeptide and the glycan antigen are associated with the outer membrane of the OMV. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine.

[0028] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a NHBA polypeptide that is associated with the outer membrane of the OMV, wherein the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

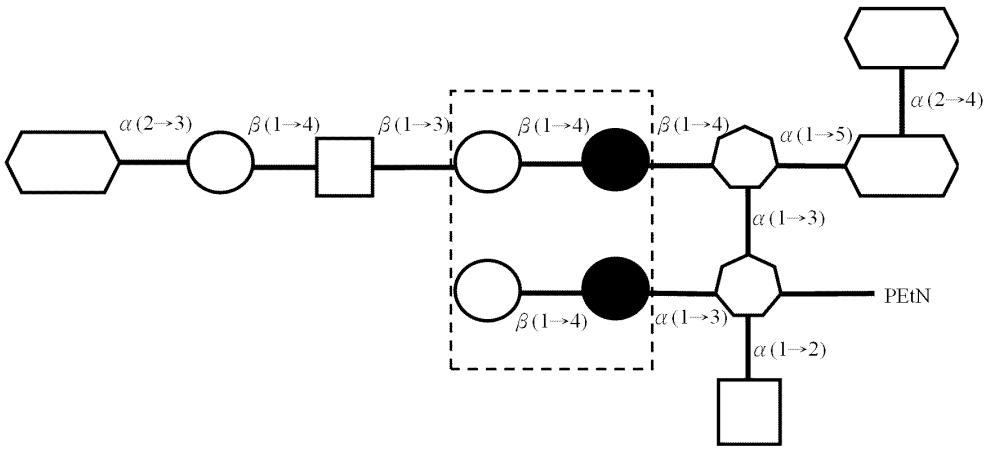
[0029] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a MsrA/B polypeptide and a glycan antigen of a pathogen, wherein the MsrA/B polypeptide and glycan

antigen are associated with the outer membrane of the OMV. In certain embodiments, the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine.

[0030] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a MsrA/B polypeptide that is associated with the outer membrane of the OMV, wherein the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments MsrA/B polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 9. In certain embodiments, the MsrA/B polypeptide comprises the amino acid sequence of SEQ ID NO: 9. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14.

[0031] In certain embodiments, the glycan antigen comprises keto-deoxyoctulosonate (KDO) as a terminal saccharide unit. In certain embodiments, the glycan antigen comprises KDOα(2→3)Gal as the terminal saccharide unit. In certain embodiments, the glycan antigen comprises KDOα(2→3)Galβ(1→4)GlcNac as the terminal saccharide unit.

[0032] In certain embodiments, the glycan antigen comprises a 2C7 epitope having the structure within the broken line square of the following structure, and is shown in **FIG. 32A**:

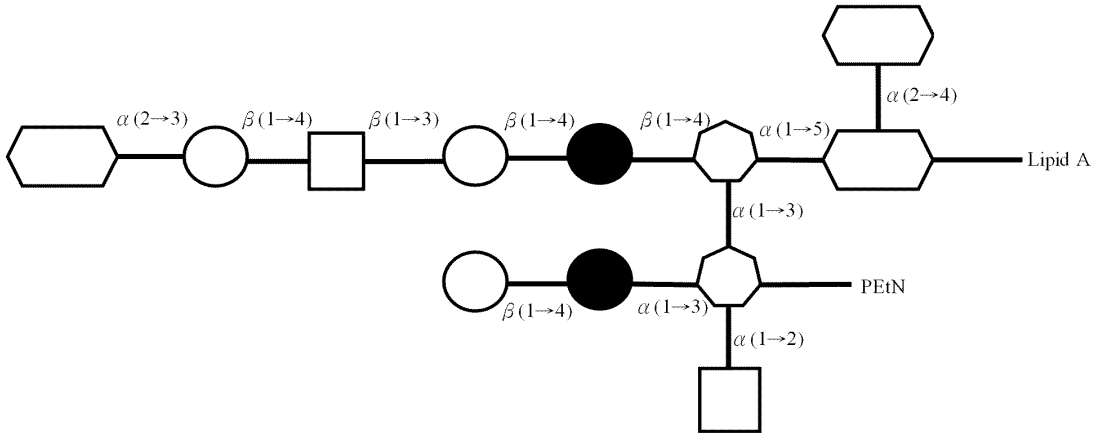


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-

heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

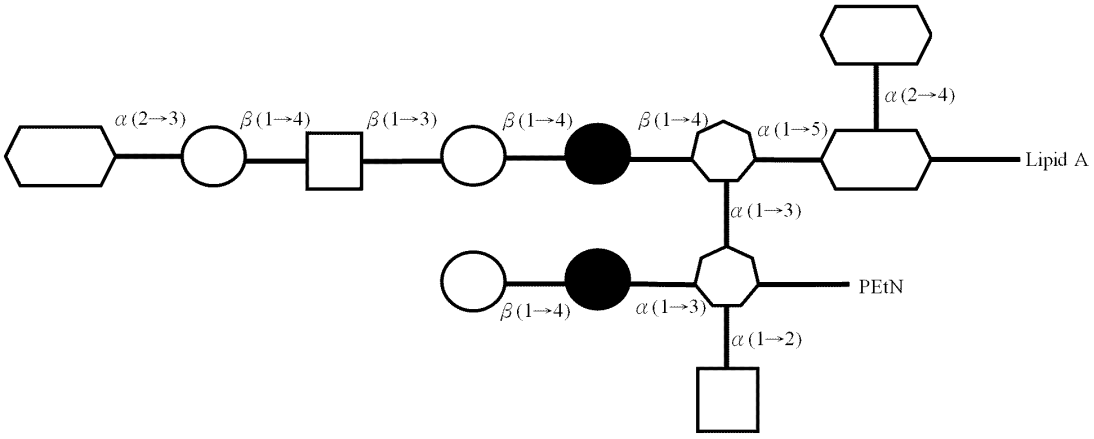
[0033] In certain embodiments, the glycan antigen is covalently conjugated to the modified lipid A.

[0034] In certain embodiments, the glycan antigen has the following structure, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

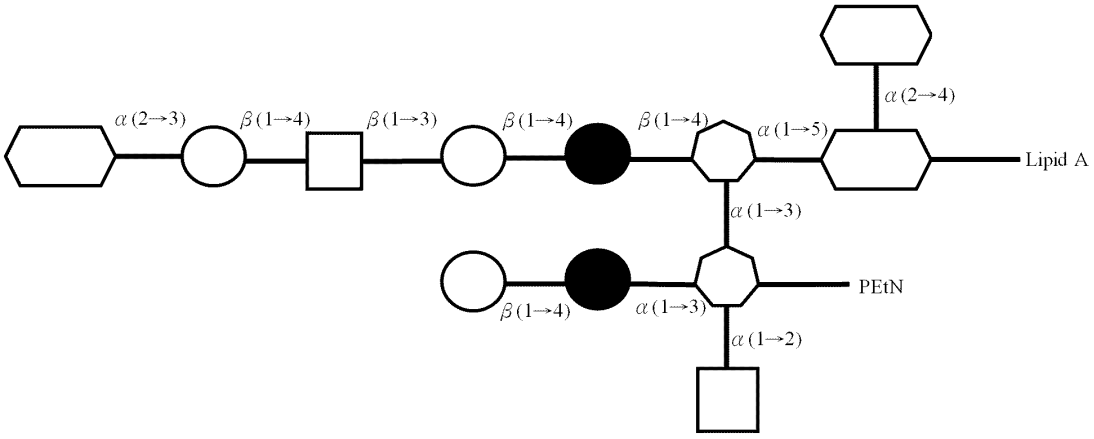
[0035] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising: (a) a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14; (b) a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7, (c) an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4, (d) a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (e) a glycan antigen has the following structure and is linked to a modified lipid A as follows, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of msbB, pagL, lpxE, and pagP; wherein the msbB, pagL, and/or lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

[0036] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising: (a) a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14; (b) a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7, (c) an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4, (d) a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (e) a glycan antigen has the following structure and is linked to a modified lipid A as follows, and is shown in **FIG. 32B**:

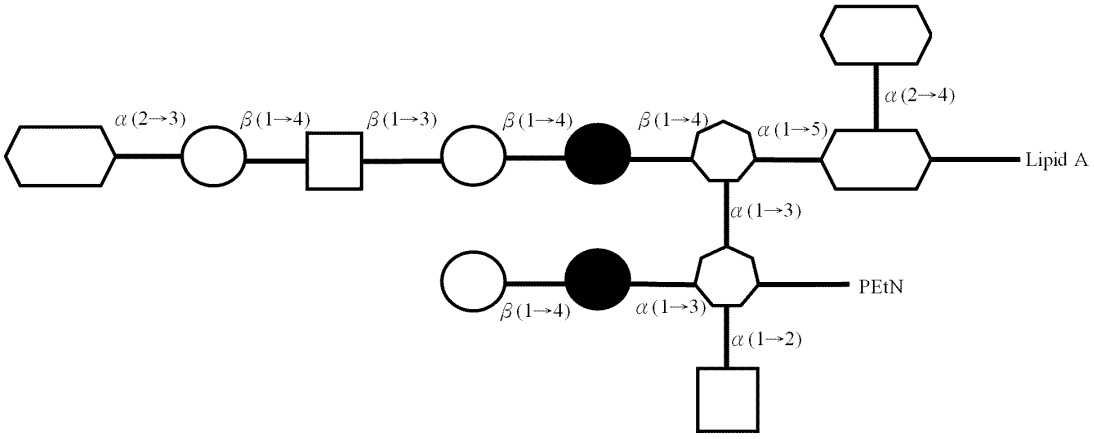


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of pagL, lpxE, and pagP;

wherein each of the pagL and lpxE is encoded by an exogenous polynucleotide of an *E. coli* that produces the OMV, wherein the *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

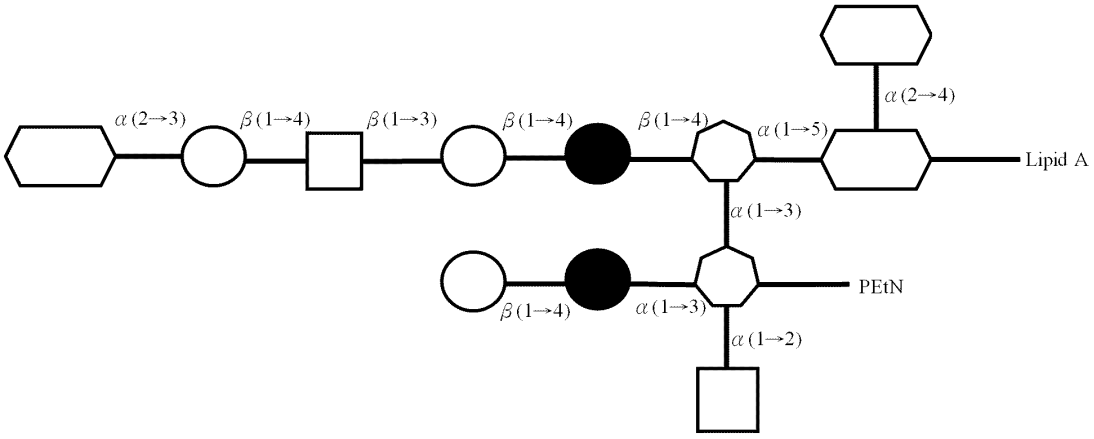
[0037] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising: (a) a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14; (b) a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7, (c) an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4, (d) a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (e) a glycan antigen has the following structure and is linked to a modified lipid A as follows, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A comprises (i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone; (ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone; (iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains; (v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and (vi) at least one lipid A species comprising a C12 acyl chain.

[0038] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising: (a) a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14; (b) a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7, (c) an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4, (d) a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (e) a glycan antigen has the following structure and is linked to a modified lipid A as follows, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine; and

(i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone, (ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone, (iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A, (iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains, (v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone, and (vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

[0039] In certain embodiments, the KDO can be recognized by antibody 6E4.

[0040] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a modified lipid A, wherein the modified lipid A comprises

(i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone;

(ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone;

(iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains;

(v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH

acyl chain at 2 position of the disaccharide backbone; and/or

(vi) at least one lipid A species comprising a C12 acyl chain.

[0041] In one aspect, the present disclosure provides an *E. coli*-derived OMV comprising a modified lipid A, wherein:

(i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone;

(ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone;

(iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A;

(iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains;

(v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and/or

(vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

[0042] In one aspect, the present disclosure provides composition comprising an OMV disclosed herein.

[0043] In one aspect, the present disclosure provides a pharmaceutical composition comprising an OMV disclosed herein, and a pharmaceutically acceptable carrier.

[0044] In one aspect, the present disclosure provides an Escherichia *coli* engineered to produce an OMV disclosed herein.

[0045] In certain embodiments, the *E. coli* comprises one or more genetic modifications to express the protein antigen. In certain embodiments, the *E. coli* comprises:

a. an exogenous polynucleotide encoding the MsrA/B polypeptide, optionally wherein the *E. coli* comprises an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, further optionally wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

b. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, optionally wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

c. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, optionally wherein the AniA signal polypeptide comprises the amino acid sequence of

SEQ ID NO: 3; and/or

d. an exogenous polynucleotide encoding the NHBA polypeptide, optionally wherein the *E. coli* comprises an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, further optionally wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

[0046] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce the modified lipid A. In certain embodiments, the *E. coli* comprises:

a. an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and/or an exogenous polynucleotide encoding lpxE;

b. genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes; and/or

c. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[0047] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce the modified lipid A. In certain embodiments, the *E. coli* comprises:

a. an exogenous polynucleotide encoding pagL and/or an exogenous polynucleotide encoding lpxE;

b. genetic disruption of *eptA* and/or *lpxT* genes; and/or

c. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[0048] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce the glycan antigen. In certain embodiments, the *E. coli* comprises:

a. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB;

b. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and/or *waaU* genes;

c. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes; and/or

d. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes.

[0049] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce the glycan antigen. In certain embodiments, the *E. coli* comprises:

a. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding lgtB, at least one exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB;

b. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and/or *waaU* genes;

c. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes; and/or

d. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes. In certain embodiments, the *E. coli* comprises two exogenous polynucleotides each encoding lgtE, two exogenous polynucleotides each encoding lst, three exogenous polynucleotides each encoding lgtA, and/or three exogenous polynucleotides each encoding lgtB.

[0050] In certain embodiments, the *E. coli* further comprises one or more genetic modifications to increase vesiculation. In certain embodiments, the *E. coli* further comprises:

a. genetic disruption of *ompA* gene;

b. genetic disruption of *lpp* gene; and/or

c. an exogenous polynucleotide encoding lpp.

[0051] In certain embodiments, the exogenous polynucleotide encoding lpp is inserted at the attλ site of the genome of the *E. coli* under the promoter Para.

[0052] In certain embodiments, the *E. coli* further comprises genetic disruption of *fliC* gene.

[0053] In certain embodiments, the *E. coli* further comprises genetic disruption of *fim* gene cluster. In certain embodiments, the *fim* gene cluster comprises *fimHGFDCIA* genes.

[0054] In one aspect, the present disclosure provides an Escherichia *coli* (*E. coli*) engineered to produce an OMV disclosed herein, wherein the *E. coli* comprises:

a. genetic modifications to express the protein antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

ii. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of

SEQ ID NO: 6;

iii. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and

iii. an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

b. genetic modifications to produce the modified lipid A, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and an exogenous polynucleotide encoding lpxE;

ii. genetic disruption of *msbB, eptA,* and *lpxT* genes; and

iii. the promoter of pagP gene of the *E. coli* being replaced by a lambda PL promoter;

c. genetic modifications to produce the glycan antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB;

ii. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes;

iii. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes; and

iv. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;

d. genetic modifications to increase vesiculation, wherein the *E. coli* comprises:

i. genetic disruption of *ompA* and *lpp* genes;

ii. an exogenous polynucleotide encoding lpp, optionally wherein the exogenous polynucleotide encoding lpp is inserted at the attλ site of the genome of the *E. coli* under the promoter Para;

e. genetic disruption of *fliC* gene; and

f. genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[0055] In one aspect, the present disclosure provides an Escherichia *coli* (*E. coli*) engineered to produce an OMV disclosed herein, wherein the *E. coli* comprises:

a. genetic modifications to express the protein antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

ii. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

iii. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and

iii. an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

b. genetic modifications to produce the modified lipid A, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding pagL and an exogenous polynucleotide encoding lpxE;

ii. genetic disruption of *eptA* and *lpxT* genes; and

iii. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

c. genetic modifications to produce the glycan antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding lgtB, at least one exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB;

ii. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes;

iii. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC,*

wzx, glf, wzy, wbbI, wbbJ, wbbL', intS, and *'wbbL* genes; and

iv. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;

d. genetic modifications to increase vesiculation, wherein the *E. coli* comprises:

i. genetic disruption of *ompA* and *lpp* genes;

ii. an exogenous polynucleotide encoding lpp;

e. genetic disruption of *fliC* gene; and

f. genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, two exogenous polynucleotides each encoding lgtE, two exogenous polynucleotides each encoding lst, three exogenous polynucleotides each encoding lgtA, three exogenous polynucleotides each encoding lgtB, and an exogenous polynucleotide encoding kdsB.

[0056] In certain embodiments, the exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide, the exogenous polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide, the exogenous polynucleotide encoding the AniA polypeptide and the AniA signal polypeptide, the exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide, and the exogenous polynucleotide encoding KdsB are expressed from a first vector.

[0057] In certain embodiments, the exogenous polynucleotide encoding lst is expressed from a second vector, wherein the expression of lst is constitutively under control of lambda phage promoter PL in the second vector.

[0058] In certain embodiments, the exogenous polynucleotide encoding msbB, the exogenous polynucleotide encoding pagL, the exogenous polynucleotide encoding lpxE, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, the exogenous polynucleotide encoding lgtE, the exogenous polynucleotide encoding lgtA, the exogenous polynucleotide encoding lgtB, the exogenous polynucleotide encoding lst, the exogenous polynucleotide encoding lpp are incorporated into the genome of the *E. coli*.

[0059] In certain embodiments, the exogenous polynucleotide encoding pagL, the exogenous polynucleotide encoding lpxE, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous

polynucleotide encoding lgtG, the at least one exogenous polynucleotide encoding lgtE, the at least one exogenous polynucleotide encoding lgtA, the at least one exogenous polynucleotide encoding lgtB, the at least one exogenous polynucleotide encoding lst, the exogenous polynucleotide encoding lpp are incorporated into the genome of the *E. coli*. In certain embodiments, one of the two exogenous polynucleotides encoding lst is expressed from a second vector; and the other one of the two exogenous polynucleotides encoding lst is incorporated into the genome of the *E. coli*.

[0060] In one aspect, the present disclosure provides a method of producing a plurality of OMVs, comprising (i) culturing an *Escherichia coli* disclosed herein under conditions suitable for producing OMVs, and (ii) purifying the OMVs produced from the *Escherichia coli*.

[0061] In one aspect, the present disclosure provides a method of inducing an immune response in a subject comprising administering to the subject an effective amount of an OMV disclosed herein or a composition disclosed herein.

4. BRIEF DESCRIPTION OF THE FIGURES

[0062] FIGS. 1A & 1B. Plasmid maps of the expression vectors pLTPro231 (**FIG. 1A**) and pLTPro302 (**FIG. 1B**) encoding the MsrA/B-AniA fusion polypeptide, MetQ polypeptide, AniA polypeptide, NHBA-MetQ fusion polypeptide, and KdsB.

[0063] FIGS. 2A & 2B. The nucleotide sequences of the expression vectors of **FIGS. 1A & 1B**. **FIG. 2A** depicts the nucleotide sequence of the expression vector pLTPro231 of **FIG. 1A** (SEQ ID NO: 66). Tac promoter is indicated by bold letters. Lac operator is indicated by bold italic letters. Ribosome binding site (RBS) is indicated by italic letters. Polynucleotide encoding MsrA/B-AniA is indicated by underlined letters. Polynucleotide encoding KdsB is indicated by wavy underlined letters. Polynucleotide encoding MetQ is indicated by dashed underlined letters. Polynucleotide encoding AniA is indicated by double underlined letters. Polynucleotide encoding NHBA-MetQ is indicated by underlined italic letters. MB T2 terminator is indicated by white letters in black background. Kanamycin resistance cassette is indicated by wavy underlined italic letters. Ori is indicated by dashed underlined italic letters. LacIq promoter is indicated by underlined bold letters. Polynucleotide encoding LacI is indicated by double underlined italic letters. **FIG. 2B** depicts the nucleotide sequence of the expression vector pLTPro302 of **FIG. 1B** (SEQ ID NO: 67). J23105 promoter is indicated by white letters in black background. Tac promoter is indicated by bold letters. Lac operator is indicated by bold italic letters. Ribosome binding site (RBS) is indicated by italic letters. Polynucleotide encoding MsrA/B-AniA is indicated by underlined letters.

Polynucleotide encoding KdsB is indicated by wavy underlined letters. Polynucleotide encoding MetQ is indicated by dashed underlined letters. Polynucleotide encoding AniA is indicated by double underlined letters. Polynucleotide encoding NHBA-MetQ is indicated by underlined italic letters. Kanamycin resistance cassette is indicated by wavy underlined italic letters. Ori is indicated by dashed underlined italic letters. LacIq promoter is indicated by underlined bold letters. Polynucleotide encoding LacI is indicated by double underlined italic letters.

[0064] FIGS. 3A-3D. Chemical structures of various lipid A species. **FIG. 3A** shows chemical structures of exemplary modified lipid A species disclosed herein. **FIG. 3B** shows the approximate percentage of the amount of each species of an exemplary modified lipid A (lipid A structure A of Examples 20 and 22) disclosed herein. **FIG. 3C** shows the approximate percentage of the amount of each species of the lipid A in the MPL® of GSK. Species in the same box could not be discriminated for quantification. Relative amount value refers to the sum of all the species in the same box. **FIG. 3D** shows the chemical structure of the wildtype *E coli.* lipid A.

[0065] FIG. 4. Scheme representing the genetic modifications and their roles for the production of the lead candidate vaccine.

[0066] FIGS. 5A-5E. Analysis of TFF purification steps. Supernatant samples from the bioreactor culturing of the lead candidate strain expressing all antigens (**FIGS. 5A-5D**, lanes 2) and from different steps of the subsequent TFF purification procedure (**FIGS. 5A-5D**, lane 3 – 7). **FIG. 5A**, SDS PAGE; **FIG. 5B**, immunoblot carried out with a mix of anti-single protein antigens antisera; **FIG. 5C**, anti-2C7 immunoblot; **FIG. 5D**, anti-KDO immunoblot carried out with the monoclonal antibody 6E4. Only a subset of Coomassie-stainable band is enriched in TFF (**FIG. 5A** lanes 2, 5, 6, 7), while another subset is washed away (**FIG. 5A** lanes 3 and 4), indicating the enrichment in OMV proteins. Protein and glycan antigens co-localize with retained fraction and they are not lost during the purification process (**FIG. 5B, FIG. 5C**, and **FIG. 5D**). **FIG. 5E** shows the annotations and samples of each lane in **FIGS. 5A-5D**.

[0067] FIGS. 6A-6F. Analysis of SEC purification. **FIG. 6A**, chromatogram of SEC purification; **FIG. 6B**, SDS PAGE analysis of the fractions; **FIGS. 6C-6E** immunoblot analysis of the fractions via anti-multi proteins immunoblot (**FIG. 6C**), anti-2C7 immunoblot (**FIG. 6D**), and anti-KDO immunoblot (**FIG. 6E**). All the proteins and the antigen co-elute in the same peak, indicating the OMV nature of the analyte. **FIG. 6F** shows the samples of each lane in **FIGS. 6B-6E**.

[0068] FIGS. 7A & 7B. Analysis of OMV purity, radius and morphology, **FIG. 7A**: SEC-HPLC profiles of OMV samples after the three purification steps (TFF1, SEC, TFF2). The peak at a

retention time of appr. 8.3 min is the OMV containing fraction, other indicated peaks are impurities. **FIG. 7B**: negative stain electron microscopy image of a purified OMV sample.

[0069] FIGS. 8A & 8B. Silver staining of the LOS from the lead candidate. **FIG. 8A** shows the bands of the LOS from the lead candidate and from a "rough" *E. coli* control, with the structures depicted nearby. **FIG. 8B** shows the expected banding pattern for complete LOS or for a "rough"-type LOS, with the structures depicted nearby. M: protein ladder used for reference; molecular weights reported.

[0070] FIGS. 9A & 9B. Graphs of the m/z values (x axis) over signal intensity (y axis) retrieved from the MALDI-MS measurement of the whole LOS. All the major peaks had a mass which correspond to the expected mass of LOS with the whole glycan devoid of the terminal KDO and different lipid A species. The corresponding species are schematized in the inlets. The lipid A structure is simplified: the two ovals represent the two glucosamine residues, the "P" on the left represents the phosphate group in position 4', and the possible acyl chains are represented by the gray curved lines.

[0071] FIGS. 10A & 10B. Graphs of the m/z values (x axis) over signal intensity (y axis) retrieved from the MALDI-MS measurement of the extracted lipid A. The distribution of species was analogous to what was observed in **FIGS. 9A & 9B**.

[0072] FIGS. 11A & 11B. Mice immunization study. **FIG. 11A**: mice immunization scheme; **FIG. 11B**: anti-protein antigens IgG titres found in sera from animals treated with the four vaccine candidates A (lead), B, C, D before immunization and after the third immunization, samples as indicated by the legend in the figure. Each circle represents a single animal. Bars indicate Geometric Mean Concentration (GMC) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA.

[0073] FIGS. 12A & 12B. Functional assays. **FIG. 12A**, Luminescent serum bactericidal assay (L-SBA) of sera from the four vaccine candidates A (lead), B, C, D as indicated by the legend compared before and after third immunization, bactericidal titer was calculated as the last dilution showing 50% killing for sera from each mouse individually. Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA. **FIG. 12B**, Gonococcal adherence assay, percentage of adherent bacteria to epithelial cells (after third immunization) relative to non-treated adhesion (pre immunization), samples as indicated by the legend in the figure.

[0074] FIG. 13. MsrA/B substrate binding inhibition assay. Percentage of blocking mediated by the antibodies after the third immunization compared to sera before immunization for the four vaccine candidates A, B, C, D, samples as indicated by the legend in the figure.

[0075] FIGS. 14A & 14B. Rabbit immunization studies for tolerability. **FIG. 14A**, rabbit immunization scheme; **FIG. 14B**: maximum temperature increase of individual animals after each of the three injections with respective vaccine candidates and vaccines.

[0076] FIG. 15. Monocyte activation test (MAT). IL-6 concentration measured by ELISA for increasing concentrations of the four vaccine candidates as well as commercially available OMV InvivoFit™ containing wildtype lipid A.

[0077] FIGS. 16A-16C. MetQ and NHBA fragments and fusion proteins investigated as gonococcal vaccine antigens. **FIG. 16A**: the full length *metQ* gene is shown (light grey arrow), as well as the MetQ (mature protein lacking the signal peptide), Met1 (N-terminal fragment of MetQ) and Met2 (C-terminal fragment of MetQ) antigens. **FIG. 16B**: the full length *nhba* gene is shown (dark gray arrow), as well as the cNHBA (C-terminal fragment of NHBA lacking the signal peptide) antigen. **FIG. 16C**: the fusions of MetQ, Met1 and Met2 (light gray) with cNHBA (dark gray) are shown. The Gly-Gly-Gly-Gly (SEQ ID NO: 68) linker used between the fused proteins is shown as an empty box.

[0078] FIGS. 17A & 17B. Structure prediction of the MetQ and NHBA antigens. **FIG. 17A**: alignment of the modelled *N. gonorrhoeae* cNHBA (dark grey) with the C-terminal domain of *N. meningitidis* NHBA (PDB 6CUJ crystal structure; light grey), and the model of *N. gonorrhoeae* MetQ, Met1 and Met2 (dark grey) with *N. meningitidis* MetQ (PDB 3GXA crystal structure; light grey). **FIG. 17B**: predicted structures of the cNHBA fusions with MetQ, Met1 or Met2 (cNHBA in light shades, fusion partners in dark shades).

[0079] FIGS. 18A & 18B. Blocking of adherence to genital tract epithelial cells by antibodies raised to the MetQ and NHBA antigens. **FIG. 18A**: blocking of adherence of *N. gonorrhoeae* strain 1291 to human male urethral epithelial cells in the presence of 1/25 dilution of sera from immunised mice, measured as % adherence relative to sera from unimmunised mice. **FIG. 18B**: blocking of adherence of *N. gonorrhoeae* strain 1291 to cervical epithelial cells in the presence of 1/25 dilution of sera from immunised mice, measured as % adherence relative to sera from unimmunised mice. 0 is the untreated (no sera) control. Student t-test p-value: * <0.5, ** <0.01, *** <0.001.

[0080] FIG. 19. ELISA titres of mouse sera raised against MetQ and NHBA fragments and fusion proteins, tested against *N. gonorrhoeae* whole cell bacteria and recombinant MetQ and NHBA proteins.

[0081] FIG. 20. Rabbit immunization study for tolerability: anti-protein antigens IgG from animals treated with low dose (LD), high dose (HD), and high dose in absence of adjuvant (HD no Alum) of the lead candidate (Lead) before immunization, after the second immunization, and after the third

immunization. Each circle represents a single animal. Bars indicate Geometric Mean Concentration (GMC) +/-95% confidence interval. ****: p<0.0001, one-way ANOVA.

[0082] FIGS. 21A & 21B. Mice immunization study. **FIG. 21A**: mice immunization scheme; **FIG. 21B**: anti-protein antigens IgG titres from animals treated with two batches of the lead candidate (Lead 1 and Lead 2), with a batch of the lead candidate without alum (Lead 2 no alum), with OMV expressing only glycan epitopes 2C7 and KDO (OMV-glyc), with OMV devoid of gonococcal antigens (OMV no Ag), and with buffer and alum (Ctrl) before immunization, after the second immunization, and after the third immunization. Each circle represents pools of two animals for post-II sera and a single animal for pre- and post-III. Bars indicate Geometric Mean Concentration (GMC) +/-95% confidence interval. ****: p<0.0001, one-way ANOVA.

[0083] FIG. 22. Mice immunization study. Anti-glycan antigens IgG titres from animals treated with two batches of the lead candidate (Lead 1 and Lead 2), with a batch of the lead candidate without alum (Lead 2 no alum), with OMV expressing only glycan epitopes 2C7 and KDO (OMV-glyc), with OMV devoid of gonococcal antigens (OMV no Ag), and with buffer and alum (Ctrl) before immunization, after the second immunization, and after the third immunization. Coating LOS is reported on top of each graph. Each circle represents a single animal. Bars indicate Geometric Mean Concentration (GMC) +/-95% confidence interval. ****: p≤ 0.0001, ***: p≤ 0.001, **: p≤ 0.01, *: p≤ 0.05, one-way ANOVA.

[0084] FIG 23. Luminescent serum bactericidal assay (L-SBA) of the same sera after third immunization. Ability of the sera to enable complement-mediated killing of gonococcal strains FA1090 and WHO-R was tested. Bactericidal titer was calculated as the last dilution showing 50% killing for sera from pools of two mice. Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: p≤ 0.0001, ***: p≤ 0.001, **: p≤ 0.01, *: p≤ 0.05, one-way ANOVA.

[0085] FIG. 24. Immunization scheme of mice immunization study with single-antigen expressing OMV.

[0086] FIG. 25. Anti-proteins Ig titres from animals treated with buffer and alum (Alum only), OMV devoid of antigens (Empty OMV), OMV carrying one protein antigen (as indicated), or soluble version of the antigen (named “control” in each protein-specific scheme). Pool of 10 animals’ sera. Endpoint titres are reported.

[0087] FIG. 26. Binding of live *Neisseria gonorrhoeae* by raised rabbit sera. Median count of cells bound by IgG from pools of post-III sera raised by immunizing rabbits with lead candidate standard dose (Lead) and middle dose (Lead-MD), OMV devoid of gonococcal antigens (OMV no Ag), and Bexsero. Rb2C7 indicates positive control monoclonal antibody used instead of raised

serum. Negative controls: gonococcal cells only (Cells), gonococcal cells and secondary antibody (Cells + 2nd Ab), sham immunization pre-immune sera (Control-pre), sham immunization post-immune sera (Control-post). Strain name is indicated for each scheme, WHO strains provided by WHO; 1263546 and 1087092 provided by JMI Labs. Measured as median fluorescent intensity.

[0088] FIGS. 27A-27C. Chemical structures of lipid A species in lipid A structure B (**FIG. 27A**), lipid A structure C (**FIG. 27B**), and lipid A structure D (**FIG. 27C**) of Examples 20 and 22.

[0089] FIG. 28. Negative stain electron microscopy image of a purified OMV sample.

[0090] FIGS. 29A-29C. Blocking of MetQ substrate binding elicited by anti-lead candidate rabbit antisera. **FIG. 29A** depicts the blocking of methionine binding to MetQ by lead candidate pre-immune and post-immune rabbit sera. Negative controls included methionine (Met only), sham immunization pre-immune sera (Control pre), and sham immunization post-immune rabbit sera (Control post). **FIG. 29B** depicts the bocking of selenomethionine binding to MetQ by lead candidate pre-immune and post-immune rabbit sera. Negative controls included selenomethionine (Met only); sham immunization pre-immune sera (Control pre), sham immunization post-immune rabbit sera (Control post). **FIG. 29C** depicts chemical structures of methionine and selenomethionine.

[0091] FIGS. 30A & 30B. Blocking of MsrA/B substrate binding elicited by anti-lead candidate rabbit antisera. **FIG. 30A** depicts the blocking value of methionine sulfoxide (MetSO) binding to MsrA/B by lead candidate pre-immune and post-immune rabbit sera. **FIG. 30B** depicts the blocking value of oxidized peptide (Pep-O) binding to MsrA/B by lead candidate pre-immune and post-immune rabbit sera.

[0092] FIGS. 31A-31D. Blocking of *N. gonorrhoeae* adherence to human cervical epithelial cells (ME180), human pharyngeal epithelial cells (FaDu), and human urethral epithelial cells (tUEC), mediated by anti-lead candidate rabbit antisera. **FIG. 31A** depicts the blocking of adherence of *Neisseria gonorrhoeae* strain 1291 (Ng 1291) or *N. gonorrhoeae* ΔNHBA (where NHBA had been knocked out) to ME180 by immunization post-immune rabbit sera of lead vaccine candidate and OMV containing only NHBA antigen. **FIG. 31B** depicts the blocking of adherence of Ng 1291 to FaDu by immunization post-immune rabbit sera of lead vaccine candidate, OMV containing only NHBA antigen, and OMV containing only NgLOS antigen. **FIG. 31C** depicts the blocking of adherence of *N. gonorrhoeae* ΔNHBA to FaDu by immunization post-immune rabbit sera of lead vaccine candidate and OMV containing only NHBA antigen. **FIG. 31D** depicts the blocking of adherence of Ng 1291 to tUEC by immunization post-immune rabbit sera of lead vaccine candidate, OMV containing only NHBA antigen, and OMV containing only NgLOS antigen.

[0093] FIGS. 32A-32C. Exemplary structures of glycan antigens disclosed herein. The 2C7 epitope comprises a structure within the broken line square of the structure shown in **FIG. 32A**. In **FIGS. 32A-32C**, the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

[0094] FIG. 33 depicts the blocking of the binding of AccA peptide to AniA by post-immune sera of rabbits immunized with the lead vaccine candidate and OMV containing only AniA antigen.

[0095] FIGS. 34A-34C. Serum immunogenicity and functionality of mice vaccinated with Lead candidate vaccine (Lead batch 1, consistency batch 1 (CB1) and consistency batch 2 (CB2)), single-antigen expressing OMV (as indicated in the figures), or control buffer. All groups were immunized with Alum as an adsorbent**. FIG. 34A** depicts IgG titres (arbitrary units, AU) against protein antigens (AniA, MetQ, NHBA and MsrA/B) measured by ECLIA in pre-immunization sera ("pre"), and in terminal bleed sera ("post-III"). Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA. **FIG. 34B** depicts IgG titres (arbitrary units, AU) against NgLOS measured by ELISA in pre-immunization sera ("pre"), and in terminal bleed sera ("post-III"). Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA. **FIG. 34C** depicts luminescent serum bactericidal titers of Lead vaccine candidate and single antigen expressing OMVs. Bactericidal titer was calculated as the last dilution showing 50% killing for sera from pools of 2 mice. Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, **: $p<0.01$, one-way ANOVA.

[0096] FIG. 35. Serum immunogenicity of rabbits vaccinated with Lead candidate vaccine (consistency batch 1 (CB1) and consistency batch 2 (CB2)), single-antigen expressing OMV (as indicated) or with control buffer. All groups were immunized with Alum as adsorbent. IgG titres (arbitrary units, AU) against protein antigens (AniA, MetQ, NHBA and MsrA/B) were measured by ECLIA in pre-immunization sera ("pre"), in sera drawn after the second immunization ("post-II"), and in terminal bleed sera ("post-III"). IgG titres (arbitrary units, AU) against NgLOS were measured by ELISA in pre-immunization sera ("pre"), and in terminal bleed sera ("post-III"). Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA.

[0097] FIG. 36. Serum immunogenicity of rats vaccinated with Lead candidate vaccine, Lead consistency batch 2 (CB2), or with control buffer. All groups were immunized with Alum as adsorbent. IgG titres (arbitrary units, AU) against protein antigens (AniA, MetQ, NHBA and MsrA/B) and NgLOS were measured by ELISA in pre-immunization sera ("pre"), in sera drawn

after the second immunization ("post-II"), and in terminal bleed sera ("post-III"). Bars indicate Geometric Mean (GM) +/-95% confidence interval. ****: $p<0.0001$, one-way ANOVA.

5. DETAILED DESCRIPTION

[0098] The present disclosure provides novel vaccines based on outer membrane vesicles (OMVs) derived from engineered *Escherichia coli* (*E. coli*). In certain embodiments, the OMVs are secreted from engineered *Escherichia coli* (*E. coli*). In certain embodiments, the *E. coli* is engineered to express on its outer membrane selective heterologous antigens of a pathogen (*e.g.*, *N. gonorrhoeae*), where the heterologous antigens are incorporated into the OMVs secreted from the *E. coli*. In certain embodiments, the heterologous antigens are associated with the outer membrane of the OMV produced by the engineered *E. coli*. In certain embodiments, the heterologous antigens are associated with the outer membrane of the OMV and are exposed on the surface of the OMVs produced by the engineered *E. coli*. In certain embodiments, the heterologous antigens are associated with the outer membrane of the OMV but are not exposed on the surface of the OMVs produced by the engineered *E. coli*. In certain embodiments, the heterologous antigens comprise glycan and/or protein antigens (*e.g.*, conserved antigens such as AniA, MetQ, MsrA/B, NHBA, KDO, and/or 2C7 epitope) of the pathogen, which can elicit broad and effective protective immunity against the pathogen regardless of its resistance profile. In certain embodiments, the *E. coli* and the OMVs derived therefrom further comprise a modified lipid A, which reduces the toxicity and improves the safety of the OMV vaccines.

[0099] As such, the presently disclosed OMVs exhibit the desirable features of eliciting broad, effective, and lasting protective immunity against the pathogens (*e.g.*, *N. gonorrhoeae*) and have superior safety profile for clinical uses. Moreover, as the OMVs are produced from *E. coli*, the OMVs do not carry various antigenic factors or protein antigens of the pathogens, and thus do not elicit immune evasion and/or suppression mechanisms of the pathogens that can reduce the protective efficacy of the OMV vaccine.

[00100] In certain embodiments, the present disclosure provides an *E. coli*-derived OMV (*e.g.*, an OMV of Section 5.1) comprising (i) at least one glycan antigen (*e.g.*, at least one glycan of Section 5.1.1), (ii) at least one protein agent (*e.g.*, at least one protein agent of Section 5.1.2), and/or (iii) a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3), wherein the at least one glycan antigen and/or the at least one protein antigen are antigens of a pathogen (*e.g.*, *N. gonorrhoeae*). In certain embodiments, the protein antigen and/or the glycan antigen is associated with the outer

membrane of the OMV. In certain embodiments, the protein antigen and/or the glycan antigen is associated with the outer membrane of the OMV and is exposed on the surface of the OMV. In certain embodiments, the protein antigen is associated with the outer membrane of the OMV but is not exposed on the surface of the OMV. In certain embodiments, the protein antigen comprises a lipidated N-terminal cysteine. In certain embodiments, the protein antigen is fused to a polypeptide comprising a lipidated N-terminal cysteine. In certain embodiments, the lipidated N-terminal cysteine assists the association of the protein antigen with the outer membrane of the OMV. In certain embodiments, the lipidated N-terminal cysteine assists the exposure of the protein antigen on the surface of the OMV. In certain embodiments, the lipidated N-terminal cysteine assists the association of the protein antigen with the outer membrane of the OMV but not the exposure of the protein antigen on the surface of the OMV. In certain embodiments, the glycan antigen is covalently conjugated to the modified lipid A that is associated with the outer membrane of the OMV.

[00101] In certain embodiments, the present disclosure further provides an engineered *E. coli* engineered to produce the presently disclosed OMV. In certain embodiments, the *E. coli* comprises (i) genetic modifications to produce at least one glycan antigen (*e.g.*, genetic modifications of Section 5.2.1), (ii) genetic modifications to produce at least one protein antigen (*e.g.*, genetic modifications of Section 5.2.2), and/or (iii) genetic modifications to produce a modified lipid A (*e.g.*, genetic modifications of Section 5.2.3). In certain embodiments, the *E. coli* further comprises genetic modifications to increase vesiculation and/or genetic disruption of *fliC* gene. In certain embodiments, the *E. coli* further comprises genetic disruption of *fim* gene cluster. In certain embodiments, the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00102] In certain embodiments, the present disclosure further provides a pharmaceutical composition comprising a presently disclosed OMV or *E. coli* (*e.g.*, a pharmaceutical composition of Section 5.3). In certain embodiments, the present disclosure further provides a method of making a presently disclosed OMV or *E. coli* (*e.g.*, a method making of Section 5.4). In certain embodiments, the present disclosure further provides a method of using a presently disclosed OMV for eliciting an immunogenic response in a subject (*e.g.*, a method using of Section 5.5). In certain embodiments, the present disclosure further provides a kit comprising a presently disclosed OMV or *E. coli* (*e.g.*, a kit of Section 5.6).

[00103] As used herein, an "antigen of a pathogen" refers to a molecule that, when administered to a subject (*e.g.*, a human or a non-human animal), can elicit a specific humoral and/or cellular immune response. In certain embodiments, the antigen is a naturally occurring

molecule of the pathogen. In certain embodiments, the antigen is derived from a naturally occurring molecule of the pathogen, for example, a mutant or a fragment of the naturally occurring molecule. In certain embodiments, the antigen is a polypeptide, a protein, a polysaccharide, a carbohydrate, a lipid, or a combination thereof. Not all antigens of a pathogen can induce a protective immunity. In certain embodiments, an antigen may induce an immune response, which is not related to the protection against the pathogen. For examples, a vaccine based on heat-killed *N. gonorrhoeae* is immunogenic and can induce an immune response. However, such immune response does not offer any protection against *N. gonorrhoeae*, *e.g.*, resisting infection or reinfection or attenuating gonorrhea or its clinical presentations.

[00104] As used herein, "protective immunity" refers to the immune system's ability to resist infection or reinfection or attenuate an infectious disease or its clinical presentation. The protective immunity can be provided by lymphocytes (*e.g.*, T cells and/or B cells) that can recognize and quickly launch attack to the pathogen after being re-exposed to the pathogen or being exposed to the pathogen for the first time after the administration of the vaccine. The protective immunity can last for months or years.

[00105] As used herein, the term "about" mean within 20%, within 15%, within 10%, within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, within 1%, or less variation of a given value or range.

[00106] The term "between" as used in a phrase as such "between A and B" or "between A-B" refers to a range including both A and B.

[00107] As used in the present disclosure and claims, the singular forms "a", "an" and "the" include plural forms unless the context clearly dictates otherwise.

5.1 Outer Membrane Vesicles (OMVs)

[00108] The present disclosure provides OMVs comprising (i) at least one glycan antigen (*e.g.*, at least one glycan of Section 5.1.1), (ii) at least one protein agent (*e.g.*, at least one protein agent of Section 5.1.2), and/or (iii) a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3). In certain embodiments, the OMVs are derived from *E. coli*. In certain embodiments, the at least one glycan antigen and/or the at least one protein antigen are antigens of a pathogen that is not *E. coli* (*e.g.*, *N. gonorrhoeae*). In certain embodiments, the at least one glycan antigen and/or the at least one protein antigen are associated with the outer membrane of the OMV.

[00109] In certain embodiments, the at least one glycan antigen and/or the at least one protein antigen can induce a protective immunity against the pathogen in a subject after the subject being administered the OMV. In certain embodiments, the protective immunity confers the subject the

ability to resist infection or reinfection of the pathogen. In certain embodiments, the protective immunity attenuates an infectious disease associated with the pathogen or its clinical presentations in the subject. In certain embodiments, the protective immunity lasts for at least about 3 months, at least about 6 months, at least about 9 months, at least about 12 months, at least about 1 year, at least about 18 months, at least about 2 years, at least 3 years, at least 4 years, or at least 5 years.

[00110] In certain embodiments, the pathogen is an agent that can cause an infectious disease. In certain embodiments, the pathogen is a virus, a bacterium, a fungus, or a parasite. In certain embodiments, the pathogen is a bacterium.

5.1.1 Glycan Antigens

[00111] In certain embodiments, an OMV disclosed herein comprises at least one glycan antigen. As used herein, a glycan antigen can be an epitope of lipooligosaccharides (LOS). In certain embodiments, the glycan antigen is an antigen of a pathogen (e.g., *N. gonorrhoeae*). In certain embodiments, the OMV comprises a KDO epitope. In certain embodiments, the OMV comprises a 2C7 epitope. In certain embodiments, the OMV comprises a KDO epitope and a 2C7 epitope. In certain embodiments, the at least one glycan antigen is associated with the outer membrane of the OMV. In certain embodiments, the glycan antigen is associated with the outer membrane of the OMV and is exposed on the surface of the OMV. In certain embodiments, the at least one glycan antigen is associated with the outer membrane of the OMV by covalently conjugated to a lipid A (e.g., a modified lipid A of Section 5.1.3), which locates on the outer membrane of the OMV.

[00112] In certain embodiments, the presently disclosed glycan antigen is produced by an engineered *E. coli* disclosed herein (*e.g., E. coli* of Sections 5.2.1 and 5.2.6), where the *E. coli* is genetically modified to knock out and knock in a number of genes relating to the synthesis of the glycan antigen (*e.g.,* genetically modifications disclosed in Sections 5.2.1 and 5.2.6), where the knock-in genes are incorporated into the genome of the *E. coli* and/or one or more vectors (*e.g.*, plasmids) transformed into the *E. coli*. In certain embodiments, the glycan antigen produced by the *E. coli* is located to and associated with the outer membrane of the *E. coli*, for example, by covalently conjugated to a lipid A associated with the outer membrane. Under suitable conditions, the *E. coli* produces and releases OMVs, where part of the outer membrane of the *E. coli* becomes the outer membrane of the OMVs, and thus the glycan antigen associated with the outer membrane of the *E. coli* is incorporated into the OMVs.

[00113] As used herein, α(2→3) represents the α-2,3-glycosidic linkage between two sugar molecules; α(2→4) represents the α-2,4-glycosidic linkage between two sugar molecules, α(1→5)

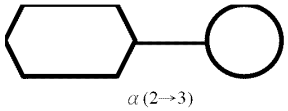
represents the α-1,5-glycosidic linkage between two sugar molecules, α(1→2) represents the α-1,2-glycosidic linkage between two sugar molecules, β(1→3) represents the β-1,3-glycosidic linkage between two sugar molecules, and β(1→4) represents the β-1,4-glycosidic linkage between two sugar molecules.

(a) KDO

[00114] In certain embodiments, a glycan antigen disclosed herein comprises a keto-deoxyoctulosonate (KDO), also known as 3-deoxy-D- manno-2-octulosonic acid, as a terminal saccharide unit.

[00115] In certain embodiments of the glycan antigen disclosed herein, the KDO further is attached to a galactose (Gal). In certain embodiments, the KDO is attached to the Gal via an α(2→3) linkage. In certain embodiments, the glycan antigen comprises KDOα(2→3)Gal.

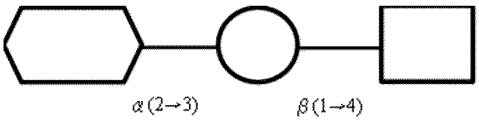
[00116] An exemplary structure of KDOα (2→3)Gal is provided below:



wherein the hexagon represents a KDO, and the white circle represents a galactose (Gal).

[00117] In certain embodiments of the glycan antigen disclosed herein, KDOα(2→3)Gal is further attached to a N-acetylglucosamine (GlcNac). In certain embodiments, KDOα(2→3)Gal is attached to a GlcNac via a β(1→4) linkage. In certain embodiments, the glycan antigen comprises KDOα(2→3)Galβ(1→4)GlcNac.

[00118] An exemplary structure of KDOα(2→3)Galβ(1→4)GlcNac is provided below:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), and the white square represents a N- acetylglucosamine (GlcNac).

[00119] In certain embodiments, the glycan antigen comprises KDOα(2→3)Gal as the terminal saccharide unit. In certain embodiments, the glycan antigen comprises KDOα(2→3)Galβ(1→4)GlcNac as the terminal saccharide unit.

[00120] In certain embodiments, the glycan antigen comprising KDO as a terminal saccharide unit further comprises a 2C7 epitope *N. gonorrhoeae*. In certain embodiments, the glycan antigen comprising KDOα(2→3)Gal as a terminal saccharide unit further comprises a 2C7 epitope *N.*

gonorrhoeae. In certain embodiments, the glycan antigen comprising KDOα(2→3)Galβ(1→4)GlcNac as a terminal saccharide unit further comprises a 2C7 epitope *N. gonorrhoeae*. Exemplary 2C7 epitope is provided in the Section 5.1.1 (b) below.

[00121] In certain embodiments, the glycan antigen comprising a KDO epitope and a 2C7 epitope is covalently conjugated to the modified lipid A. In certain embodiments, the glycan antigen comprising KDOα(2→3)Gal and a 2C7 epitope is covalently conjugated to the modified lipid A. In certain embodiments, the glycan antigen comprising KDOα(2→3)Galβ(1→4)GlcNac and a 2C7 epitope is covalently conjugated to the modified lipid A. Exemplary modified lipid A is provided in the Section 5.1.3 below.

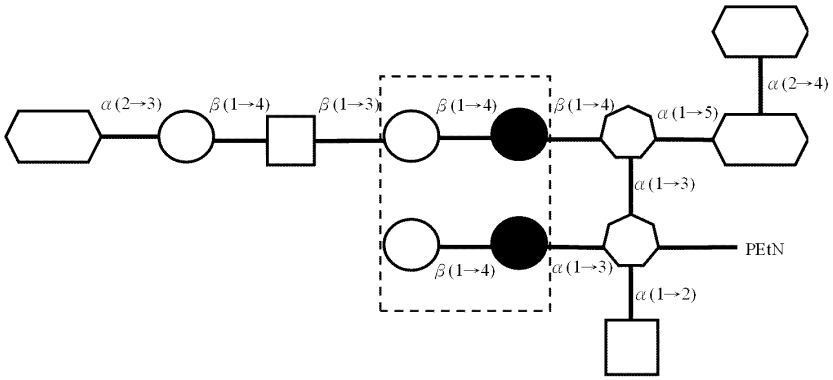
[00122] In certain embodiments, the KDO can be recognized by antibody 6E4 (*see* Jen FE et al., mBio, 2021;12(2):e03666-20)

(b) 2C7

[00123] In certain embodiments, a glycan antigen disclosed herein comprises a 2C7 epitope. In certain embodiments, the glycan antigen comprises a 2C7 epitope of *N. gonorrhoeae*.

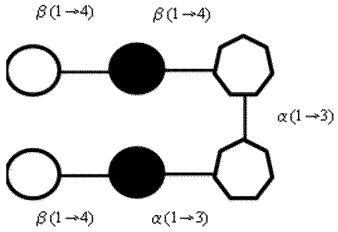
[00124] 2C7 epitope is a conserved oligosaccharide (OS) structure, a part of lipooligosaccharide (LOS) on *N. gonorrhoeae*. This structure is recognized by a murine monoclonal antibody called 2C7. Human antibodies against the 2C7 epitope mediate complement-dependent bacterial killing and opsonophagocytosis. See Gulati *et al*, *PLOS Pathogens* (2013);8:e1003559 for a detailed discussion of the 2C7 epitope, the content of which is incorporated by reference herein.

[00125] In certain embodiments of the glycan antigen disclosed herein, the 2C7 epitope comprises two lactose branches, wherein each lactose branch comprises a galactose (Gal) and a glucose (Glc). In certain embodiments, the Gal is attached to the Glc. In certain embodiments, the Gal is attached to the Glc via a β(1→4) linkage. In certain embodiments, the 2C7 epitope comprises a first Galβ(1→4)Glc and a second Galβ(1→4)Glc. In certain embodiments, the 2C7 epitope comprises a structure within the broken line square of the structure provided below, and is shown in **FIG. 32A**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

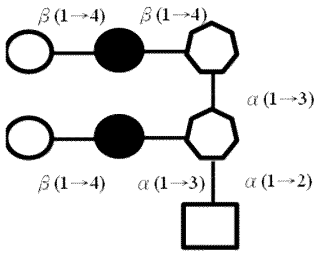
[00126] In certain embodiments of the 2C7 epitope disclosed herein, the first Galβ(1→4)Glc is further attached to a first L-glycero-D-manno-heptose (Hep1). In certain embodiments of the 2C7 epitope disclosed herein, the first Galβ(1→4)Glc is further attached to Hep1 via a β(1→4) linkage as Galβ(1→4)Glcβ(1→4)Hep1. In certain embodiments of the 2C7 epitope disclosed herein, the second Galβ(1→4)Glc is further attached to a second L-glycero-D-manno-heptose (Hep2). In certain embodiments of the 2C7 epitope disclosed herein, the second Galβ(1→4)Glc is further attached to Hep2 via an α(1→3) linkage as Galβ(1→4)Glcα(1→3)Hep2. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises Galβ(1→4)Glcβ(1→4)Hep1 and Galβ(1→4)Glcα(1→3)Hep2, wherein Hep1 is attached to Hep2 via an α(1→3) linkage. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises an exemplary structure provided below:



wherein the white circle represents a galactose (Gal), the black circle represents a glucose (Glc), and the heptagon represents a L-glycero-D-manno-heptose (Hep).

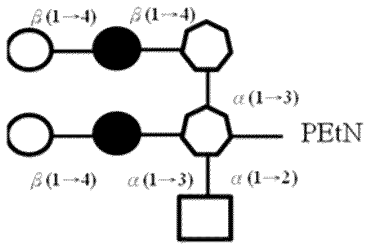
[00127] In certain embodiments of the 2C7 epitope disclosed herein, the second L-glycero-D-manno-heptose (Hep2) is further attached to a N- acetylglucosamine (GlcNac). In certain

embodiments, Hep2 is further attached to a GlcNac via an α(1→2) linkage. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises Galβ(1→4)Glcβ(1→4)Hep1 and Galβ(1→4)Glcα(1→3)Hep2, wherein Hep1 is attached to Hep2 via an α(1→3) linkage, and Hep2 is further attached to GlcNac via an α(1→2) linkage. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises an exemplary structure provided below:



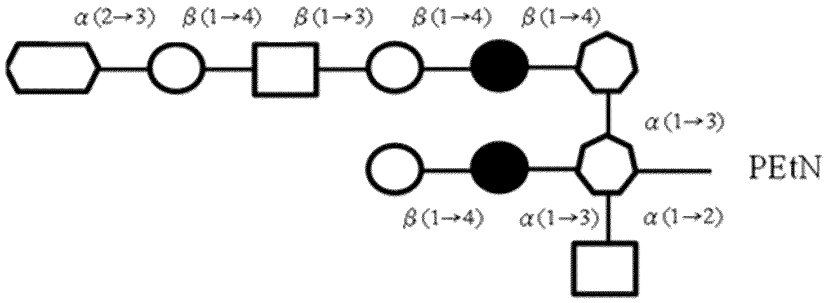
wherein the white circle represents a galactose (Gal), the black circle represents a glucose (Glc), the heptagon represents a L-glycero-D-manno-heptose (Hep), and the white square represents a N-acetylglucosamine (GlcNac).

[00128] In certain embodiments of the 2C7 epitope disclosed herein, the second L-glycero-D-manno-heptose (Hep2) is further attached to a phosphoethanolamine (PEtN). In certain embodiments of the 2C7 epitope disclosed herein, the second L-glycero-D-manno-heptose (Hep2) is further attached to a N-acetylglucosamine (GlcNac) and a PEtN. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises Galβ(1→4)Glcβ(1→4)Hep1 and Galβ(1→4)Glcα(1→3)Hep2, wherein Hep1 is attached to Hep2 via an α(1→3) linkage, and Hep2 is further attached to GlcNac via an α(1→2) linkage and a PEtN. In certain embodiments, a glycan antigen comprising the 2C7 epitope disclosed herein comprises an exemplary structure provided below:



wherein the white circle represents a galactose (Gal), the black circle represents a glucose (Glc), the heptagon represents a L-glycero-D-manno-heptose (Hep), the white square represents a N-acetylglucosamine (GlcNac), and the PEtN represents a phosphoethanolamine.

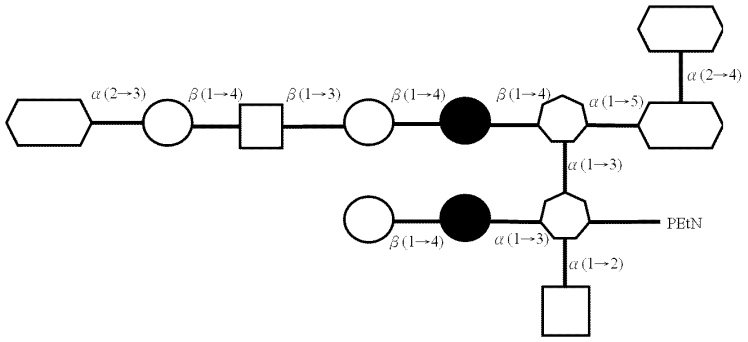
[00129] In certain embodiments, the 2C7 epitope is further attached to the KDO as provided in the Section 5.1.1(a) above. In certain embodiments, the 2C7 epitope is further attached to the terminal saccharide unit as provided in the Section 5.1.1(a) above. In certain embodiments of the glycan antigen disclosed herein, the 2C7 epitope is further attached to KDOα(2→3)Galβ(1→4)GlcNac as provided in the Section 5.1.1(a) above. In certain embodiments of the glycan antigen disclosed herein, the first Galβ(1→4)Glc of the 2C7 epitope is further attached to KDOα(2→3)Galβ(1→4)GlcNac as provided in the Section 5.1.1(a) above. In certain embodiments, KDOα(2→3)Galβ(1→4)GlcNac is attached to the first Galβ(1→4)Glc of the 2C7 epitope via a β(1→3) linkage. In certain embodiments, the glycan antigen comprises KDOα(2→3)Galβ(1→4)GlcNac as the terminal saccharide unit and a 2C7 epitope. In certain embodiments, the glycan antigen comprises an exemplary structure provided below, and is shown in **FIG. 32C**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

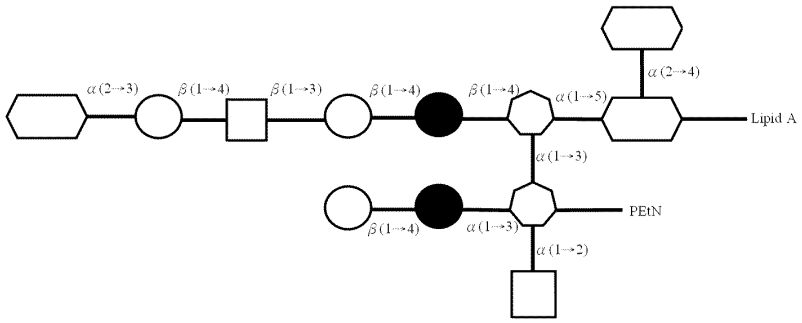
[00130] In certain embodiments, KDOα(2→3)Galβ(1→4)GlcNac and the 2C7 epitope is further attached to a second KDO via an α(1→5) linkage. In certain embodiments, the second KDO is further attached to a third KDO via an α(2→4) linkage. In certain embodiments, the third KDO is further attached to PEtN.

[00131] In certain embodiments, the glycan antigen comprises a KDOα(2→3)Galβ(1→4)GlcNac, a 2C7 epitope, a second KDO, and a third KDO. In certain embodiments, a glycan antigen disclosed herein comprises an exemplary structure provided below, and is shown in **FIG. 32A**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

[00132] In certain embodiments, the glycan antigen is further covalently conjugated to a modified lipid A. In certain embodiments, the glycan antigen comprises an exemplary structure is provided below, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

5.1.2 Protein Antigens

[00133] In certain embodiments, an OMV disclosed herein comprises at least one protein antigen. In certain embodiments, the protein antigen is an antigen of a pathogen (*e.g.*, *N. gonorrhoeae*). In certain embodiments, the OMV comprises an AniA polypeptide, a MetQ polypeptide, a MsrA/B polypeptide, and/or a NHBA polypeptide. In certain embodiments, the protein antigen is associated with the outer membrane of the OMV. In certain embodiments, the protein antigen is associated with the outer membrane of the OMV and is exposed on the surface of

the OMV. In certain embodiments, the protein antigen is associated with the outer membrane of the OMV but is not exposed on the surface of the OMV. In certain embodiments, the protein antigen comprises a lipidated N-terminal cysteine. In certain embodiments, the protein antigen is fused to a polypeptide comprising a lipidated N-terminal cysteine. In certain embodiments, the lipidated N-terminal cysteine assists the association of the protein antigen with the outer membrane of the OMV. In certain embodiments, the lipidated N-terminal cysteine assists the exposure of the protein antigen on the surface of the OMV. In certain embodiments, the lipidated N-terminal cysteine assists the association of the protein antigen with the outer membrane of the OMV but not the exposure of the protein antigen on the surface of the OMV.

[00134] In certain embodiments, the presently disclosed protein antigen is produced by an engineered *E. coli* disclosed herein (*e.g.*, *E. coli* of Sections 5.2.2 and 5.2.6), where the *E. coli* is genetically modified and transformed with one or more vectors (*e.g.*, plasmids) that express the protein antigen (*e.g.*, genetically modifications disclosed in Sections 5.2.2 and 5.2.6). In certain embodiments, the protein antigen produced by the *E. coli* is located to and associated with the outer membrane of the *E. coli*, for example, through lipidated N-terminal cysteine. Under suitable conditions, the *E. coli* produces and releases OMVs, where part of the outer membrane of the *E. coli* becomes the outer membrane of the OMVs, and thus the protein antigen associated with the outer membrane of the *E. coli* is incorporated into the OMVs.

(a) AniA Polypeptides

[00135] In certain embodiments, an OMV disclosed herein comprises a Neisseria nitrite reductase (AniA) polypeptide. Exemplary AniA polypeptides are described in International Patent Publication WO2010022462, the contents of which are incorporated by reference herein in their entireties.

[00136] AniA is a nitrate reductase essential for anaerobic growth and biofilm formation. AniA is glycosylated in the carboxy terminal region. AniA is known to be an immunogenic outer membrane protein, and glycosylation of this protein with the same phase variable structure that is found on pilin may serve an immune-evasion role. Removal or modification of the glycan or the glycosylated region may lead to a non-native, protective immune response.

[00137] In certain embodiments, provided are AniA polypeptides, which can be used as described herein in compositions, methods and uses for eliciting an immune response to N. gonorrhoeae in a subject, for immunizing a subject against N. gonorrhoeae, and for the prevention and treatment of an infection and/or disease caused by N. gonorrhoeae.

[00138] In certain embodiments, the AniA polypeptide is derived from a wild-type full length AniA polypeptide from *N. gonorrhoeae*. The amino acid sequence of an exemplary full length AniA polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 1 (with signal peptide). The amino acid sequence of an exemplary full length AniA polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 50 (without signal peptide).

[00139] In certain embodiments, the AniA polypeptide comprises a fragment of the full length AniA polypeptide. In certain embodiments, the AniA polypeptide comprises a fragment of the full length AniA polypeptide, including those truncated at the C-terminus and/or N-terminus of the full length AniA polypeptide. In certain embodiments, the AniA polypeptide lacks at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or more amino acids at the C-terminus and/or N-terminus compared to the full length AniA polypeptide.

[00140] In certain embodiments, the AniA polypeptide comprises a fragment lacking the C-terminal region of the full length AniA polypeptide. In certain embodiments, the AniA polypeptide lacks 56 amino acids at the C-terminus compared to the full length AniA polypeptide. In certain embodiments, the AniA polypeptide comprises amino acids 1- 346 of SEQ ID NO: 1.

[00141] In certain embodiments, the AniA polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2. In certain embodiments, the AniA polypeptide disclosed herein comprises an AniA signal peptide. In certain embodiments, the AniA signal peptide comprises the amino acid sequence of SEQ ID NO: 3.

[00142] In certain embodiments, the AniA signal peptide is removed during post-translational modifications. In certain embodiments, the AniA polypeptide lacks all or a portion of the AniA signal sequence, *i.e.*, truncated at the N-terminus. In certain embodiments, the AniA polypeptide comprises amino acids 19-346 of SEQ ID NO: 1. In certain embodiments, the AniA polypeptide comprises amino acids 19-346 of SEQ ID NO: 2. In certain embodiments, the AniA polypeptide lacks the AniA signal peptide. In certain embodiments, the AniA polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 4 (without signal peptide).

[00143] In certain embodiments, the AniA polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, removal of the signal peptide is coupled with the lipidation process. In certain embodiments, removal of the signal peptide and the lipidation process are carried out by *E. coli* SEC-SPII pathway. In certain embodiments, the lipidation process is coupled with the export of the protein and results in the anchoring of the protein in *E. coli* outer membrane.

An exemplary lipidation process is described in Nakayama et al., FEBS J (2012);279(23):4247-68, the content of which is incorporated by reference in its entirety. In certain embodiments, the anchoring of the AniA polypeptide in *E. coli* outer membrane results in the incorporation of the AniA polypeptide in the OMV outer membrane through the releasing of the OMV from the *E. coli*.

[00144] In certain embodiments, the AniA polypeptide comprises amino acid substitutions or deletions, which do not change the immunogenic activity of the AniA polypeptide. In certain embodiments, the AniA polypeptide comprises amino acid substitutions or deletions, which improve the immunogenic activity of the AniA polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 1, SEQ ID NO: 50, SEQ ID NO: 2, or SEQ ID NO: 4 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 1, SEQ ID NO: 50, SEQ ID NO: 2, or SEQ ID NO: 4. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 1, SEQ ID NO: 50, SEQ ID NO: 2, or SEQ ID NO: 4 may be modified (*e.g.*, by amino acid substitution, deletion) so that the variant improves the immunogenicity of SEQ ID NO: 1, SEQ ID NO: 50, SEQ ID NO: 2, or SEQ ID NO: 4.

[00145] In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 1. In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 50. In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 2. In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 4.

[00146] In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 38. In certain embodiments, the AniA polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 38.

[00147] In certain embodiments, the AniA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO: 44. In certain embodiments, the AniA polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 44.

(b) MetQ Polypeptides

[00148] In certain embodiments, an OMV disclosed herein comprises a methionine ABC transporter substrate-binding lipoprotein (MetQ) polypeptide.

[00149] MetQ is a multifunctional lipoprotein on the bacterial surface that is involved in methionine transport and *N. gonorrhoeae* adhesion to cervical epithelial cells and monocytes. In certain embodiments, provided are MetQ polypeptides, which can be used as described herein in compositions, methods and uses for eliciting an immune response to *N. gonorrhoeae* in a subject, for immunizing a subject against *N. gonorrhoeae*, and for the prevention and treatment of an infection and/or disease caused by *N. gonorrhoeae*.

[00150] In certain embodiments, a MetQ polypeptide disclosed herein is derived from a wild-type full length MetQ polypeptide from *N. gonorrhoeae*. The amino acid sequence of an exemplary full length MetQ polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 5 (with signal peptide). The amino acid sequence of an exemplary full length MetQ polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 7 (without signal peptide).

[00151] In certain embodiments, the MetQ polypeptide comprises a fragment of the full length MetQ polypeptide. In certain embodiments, the MetQ polypeptide comprises a fragment of the full length MetQ polypeptide, including those truncated at the C-terminus and/or N-terminus of the full length MetQ polypeptide. In certain embodiments, the MetQ polypeptide lacks at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31,

32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or more amino acids at the C-terminus and/or N-terminus compared to the full length MetQ polypeptide.

[00152] In certain embodiments, the MetQ polypeptide comprises the full length MetQ polypeptide. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 5. In certain embodiments, the MetQ polypeptide comprises a MetQ signal peptide. In certain embodiments, the MetQ signal peptide comprises the amino acid sequence of SEQ ID NO: 6.

[00153] In certain embodiments, the MetQ signal peptide is removed during post-translational modifications. In certain embodiments, the MetQ polypeptide lacks all or a portion of the MetQ signal sequence, *i.e.*, truncated at the N-terminus. In certain embodiments, the MetQ polypeptide comprises amino acids 20-288 SEQ ID NO: 5.

[00154] In certain embodiments, the MetQ polypeptide lacking the MetQ signal peptide comprises the amino acid sequence set forth in SEQ ID NO: 7.

[00155] In certain embodiments, the MetQ polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, removal of the signal peptide is coupled with the lipidation process. In certain embodiments, removal of the signal peptide and the lipidation process are carried out by *E. coli* SEC-SPII pathway. In certain embodiments, the lipidation process is coupled with the export of the protein and results in the anchoring of the protein in *E. coli* outer membrane. An exemplary lipidation process is described in Nakayama et al., FEBS J (2012);279(23):4247-68, the content of which is incorporated by reference in its entirety. In certain embodiments, the anchoring of the MetQ polypeptide in *E. coli* outer membrane results in the incorporation of the MetQ polypeptide in the OMV outer membrane through the releasing of the OMV from the *E. coli*.

[00156] In certain embodiments, the MetQ polypeptide comprises amino acid substitutions or deletions, which do not change the immunogenic activity of the MetQ polypeptide. In certain embodiments, the MetQ polypeptide comprises amino acids substitutions or deletions, which improve the immunogenic activity of the MetQ polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 5, or SEQ ID NO: 7 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 5, or SEQ ID NO: 7. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 5, or SEQ ID NO: 7 may be modified (*e.g.*, by amino acid substitution, deletion) so that the variant improves the immunogenicity of SEQ ID NO: 5, or SEQ ID NO: 7.

[00157] In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 5. In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 7.

[00158] In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 39. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 39.

[00159] In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO: 45. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 45.

[00160] In certain embodiments, the MetQ polypeptide is a N-terminal amino acid fragment of the full length MetQ polypeptide. In certain embodiments, the MetQ polypeptide is an N-terminal, 100-110 amino acid fragment of the full length MetQ polypeptide (not including the signal peptide, *e.g.*, of SEQ ID NO: 7). In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 57. In certain embodiments, the

MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 57. In certain embodiments, the MetQ polypeptide is designated as "Met1".

[00161] In certain embodiments, the MetQ polypeptide is a C-terminal amino acid fragment of the full length MetQ polypeptide. In certain embodiments, the MetQ polypeptide is a C-terminal, 160-170 amino acid fragment of the full length MetQ polypeptide (not including the signal peptide, *e.g.*, of SEQ ID NO: 7). In certain embodiments, the MetQ polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 58. In certain embodiments, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 58. In certain embodiments, the MetQ polypeptide is designated as "Met2".

[00162] In certain embodiments, the MetQ polypeptide disclosed herein is fused to a NHBA polypeptide to form a NHBA-MetQ fusion polypeptide. In certain embodiments, the NHBA polypeptide is a NHBA polypeptide disclosed in Section 5.1.2(d). In certain embodiments, the NHBA-MetQ fusion polypeptide is a NHBA-MetQ fusion polypeptide disclosed in Section 5.1.2(d).

(c) MsrA/B Polypeptides

[00163] In certain embodiments, an OMV disclosed herein comprises a methionine sulfoxide reductases (MsrA/B) polypeptide. Exemplary MsrA/B polypeptides are described in International Patent Publication WO2020124159, the contents of which are incorporated by reference herein in their entireties.

[00164] MsrA/B plays an important role in protecting *N. gonorrhoeae* from oxidative damage (Skaar et ai, 2002, Proc Natl Acad Sci U S A 99(15), 10108-10113), by catalyzing the reduction of methionine sulfoxide residues Met(O) back to methionine (Met) (Lowther et al., 2002, Nat Struct Biol 9(5), 348- 352; and Brot et al., 2006, J Biol Chem 281(43), 32668-32675). MsrA/B from *N. gonorrhoeae* is present, highly conserved and expressed in all *N. gonorrhoeae* strains investigated in the present studies and is immunogenic.

[00165] In certain embodiments, provided are MsrA/B polypeptides, which can be used as described herein in compositions, methods and uses for eliciting an immune response to *N. gonorrhoeae* in a subject, for immunizing a subject against *N. gonorrhoeae*, and for the prevention and treatment of an infection and/or disease caused by *N. gonorrhoeae*.

[00166] In certain embodiments, the MsrA/B polypeptide is derived from a wild-type full length MsrA/B polypeptide from *N. gonorrhoeae*. The amino acid sequence of an exemplary full length MsrA/B polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 8 (with signal peptide). The amino acid sequence of an exemplary full length MsrA/B polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 51 (without signal peptide).

[00167] In certain embodiments, the MsrA/B polypeptide comprises a fragment of the full length MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide comprises a fragment of the full length MsrA/B polypeptide, including those truncated at the N-terminus of the full length MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide lacks at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or more amino acids at the N-terminus compared to the full length MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide lacks 34 amino acids at the N-terminus compared to the full length MsrA/B polypeptide.

[00168] In certain embodiments, the MsrA/B polypeptide comprises a fragment of the full length MsrA/B polypeptide, including those truncated at the C-terminus of the full length MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide lacks at least about 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160,170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340 or more amino acids at the N-terminus compared to the full length MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide lacks 340 amino acids at the C-terminus compared to the full length MsrA/B polypeptide.

[00169] In certain embodiments, the MsrA/B polypeptide comprises an immunogenic fragment of the full length MsrA/B polypeptide, including those that comprise all or a portion of the thioredoxin domain. In certain embodiments, the MsrA/B polypeptide comprises amino acid 35-182 of SEQ ID NO: 8. In certain embodiments, the MsrA/B polypeptide disclosed herein comprises the amino acid sequence set forth in SEQ ID NO: 9.

[00170] In certain embodiments, the MsrA/B polypeptide comprises amino acid substitutions or deletions, which do not change the immunogenic activity of the MsrA/B polypeptide. In certain embodiments, the MsrA/B polypeptide comprises amino acid substitutions or deletions, which improve the immunogenic activity of the MsrA/B polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 8, SEQ ID NO: 51, or SEQ ID NO: 9 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 8, SEQ ID NO: 51, or SEQ ID NO: 9. In certain embodiments, one or more of the amino

acid residues of SEQ ID NO: 8, SEQ ID NO: 51, or SEQ ID NO: 9 may be modified (*e.g.*, by amino acid substitution, deletion) so that the variant improves the immunogenicity of SEQ ID NO: 8, SEQ ID NO: 51, or SEQ ID NO: 9.

[00171] In certain embodiments, the MsrA/B polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 8. In certain embodiments, the MsrA/B polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 51. In certain embodiments, the MsrA/B polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 9.

[00172] In certain embodiments, the MsrA/B polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 46. In certain embodiments, the MsrA/B polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 46.

[00173] In certain embodiments, the MsrA/B polypeptide is fused to an AniA polypeptide to form a MsrA/B-AniA fusion polypeptide. In certain embodiments, the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of an AniA polypeptide to form a MsrA/B-AniA fusion polypeptide. In certain embodiments, the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide. In certain embodiments, the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine. In certain embodiments, the lipidated AniA assists the association of the MsrA/B polypeptide to the outer membrane of the OMV. In certain embodiments, the lipidated AniA assists the exposure of the MsrA/B polypeptide to surface of the OMV. In certain embodiments, the lipidated AniA assists the

association of the MsrA/B polypeptide to the outer membrane of the OMV but not the exposure of the MsrA/B polypeptide to surface of the OMV.

[00174] In certain embodiments of the MsrA/B-AniA fusion polypeptide provided herein, the MsrA/B polypeptide comprises the amino acid sequence of SEQ ID NO: 9.

[00175] In certain embodiments of the MsrA/B-AniA fusion polypeptide provided herein, the AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 10.

[00176] In certain embodiments of the MsrA/B-AniA fusion polypeptide provided herein, the AniA polypeptide comprises an AniA signal peptide. In some embodiment, the AniA signal peptide comprises the amino acid sequence of SEQ ID NO: 3. In certain embodiments of the MsrA/B-AniA fusion polypeptide provided herein, the AniA polypeptide fused to the MsrA/B polypeptide lacks all or a portion of the AniA signal sequence, *i.e.*, truncated at the N-terminus. In certain embodiments, the AniA polypeptide lacking the AniA signal peptide disclosed herein comprises the amino acid sequence of SEQ ID NO: 12.

[00177] In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 13. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an AniA signal peptide. In some embodiment, the AniA signal peptide comprises the amino acid sequence of SEQ ID NO: 3.

[00178] In certain embodiments, the AniA signal peptide is removed during post-translational modifications. In certain embodiments, the MsrA/B-AniA fusion polypeptide provided herein lacks all or a portion of the AniA signal sequence, i.e., truncated at the N-terminus. In certain embodiments, the MsrA/B-AniA fusion polypeptide provided herein comprises amino acids 19-199 of SEQ ID NO: 13. In certain embodiments, the MsrA/B-AniA fusion polypeptide lacking the AniA signal peptide disclosed herein comprises the amino acid sequence set forth in SEQ ID NO: 14.

[00179] In certain embodiments of the MsrA/B-AniA fusion polypeptide provided herein, the cysteine at the N-terminal of the MsrA/B-AniA fusion polypeptide is lipidated. In certain embodiments, the signal peptide removal is coupled with the lipidation process. In certain embodiments, the signal peptide removal and the lipidation process are carried out by *E. coli* SEC-SPII pathway. In certain embodiments, the lipidation process is as well coupled with the export of the protein and results in the anchoring of the protein in *E. coli* outer membrane. An exemplary lipidation process is described in Nakayama et al., FEBS J (2012);279(23):4247-68, the content of which is incorporated by reference in its entirety. In certain embodiments, the anchoring of the MsrA/B-AniA fusion polypeptide in *E. coli* outer membrane results in the incorporation of the

MsrA/B-AniA fusion polypeptide in the OMV outer membrane through the releasing of the OMV from the *E. coli*.

[00180] In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises amino acid substitutions or deletions, which do not change the immunogenic activity of the MsrA/B-AniA fusion polypeptide. In certain embodiments the MsrA/B-AniA fusion polypeptide comprises amino acid substitutions or deletions, which improve the immunogenic activity of the MsrA/B-AniA fusion polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 13 or SEQ ID NO: 14 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 13 or SEQ ID NO: 14. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 13 or SEQ ID NO: 14 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant improves the immunogenicity of SEQ ID NO: 13 or SEQ ID NO: 14.

[00181] In certain embodiments of the MsrA/B-AniA fusion comprises the MsrA/B polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 9, and the AniA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 10. In certain embodiments of the MsrA/B-AniA fusion comprises the MsrA/B polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 9, and the AniA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 12.

[00182] In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at

least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 13. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 14.

[00183] In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 40. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 40.

[00184] In certain embodiments, the MsrA/B-AniA fusion comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO: 47. In certain embodiments, the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 47.

(d) *NHBA Polypeptides*

[00185] In certain embodiments, an OMV disclosed herein comprises a Neisserial heparin binding antigen (NHBA) polypeptide. Exemplary NHBA polypeptides are described in International Patent Publication WO2021102505, the contents of which are incorporated by reference herein in their entireties.

[00186] NHBA is a lipoprotein that binds to heparin and chondroitin sulfate and is highly conserved in *N. gonorrhoeae* strains (97%-100% identity), and may be involved in adhesion of gonococcus to host epithelial cells.

[00187] In certain embodiments, provided are NHBA polypeptides, which can be used as described herein in compositions, methods and uses for eliciting an immune response to *N.*

gonorrhoeae in a subject, for immunizing a subject against *N. gonorrhoeae*, and for the prevention and treatment of an infection and/or disease caused by *N. gonorrhoeae*.

[00188] In certain embodiments, the NHBA polypeptide disclosed herein is derived from a wild-type full length NHBA polypeptide. An exemplary full length NHBA polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 15. The amino acid sequence of an exemplary full length NHBA polypeptide from *N. gonorrhoeae* is set forth in SEQ ID NO: 52 (without signal peptide).

[00189] In certain embodiments, the NHBA polypeptide disclosed herein comprises a fragment of the full length NHBA polypeptide from *N. gonorrhoeae*. In certain embodiments, the NHBA polypeptide disclosed herein comprises a fragment of a full length NHBA polypeptide, including those truncated at the N-terminus of the full length NHBA polypeptide. In certain embodiments, the NHBA polypeptide lacks at least about 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160,170, 180, 190, 200, 210, 220, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 270 or more amino acids at the N-terminus compared to a full length NHBA polypeptide. In certain embodiments, the NHBA polypeptide lacks 243 amino acids at the N-terminus compared to a full length NHBA polypeptide.

[00190] In certain embodiments, the NHBA polypeptide disclosed herein comprises amino acids corresponding to amino acid 244-426 of SEQ ID NO: 15. In certain embodiments, the NHBA polypeptide disclosed herein comprises the amino acid sequence set forth in SEQ ID NO: 16.

[00191] In certain embodiments of the NHBA polypeptide comprises amino acid substitutions or deletions, which do not change immunogenic activity of the NHBA polypeptide. In certain embodiments, the NHBA polypeptide comprises amino acid substitutions or deletions, which improve the immunogenic activity of the NHBA polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 15, SEQ ID NO: 52, or SEQ ID NO: 16 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 15, SEQ ID NO: 52, or SEQ ID NO: 16. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 15, SEQ ID NO: 52, or SEQ ID NO: 16 may be modified (*e.g.*, by amino acid substitution, deletion) so that the variant improves the immunogenicity of SEQ ID NO: 15, SEQ ID NO: 52, or SEQ ID NO: 16.

[00192] In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence

identity with the amino acid sequence of SEQ ID NO: 15. In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with the amino acid sequence of SEQ ID NO: 52. In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with the amino acid sequence of SEQ ID NO: 16.

[00193] In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO: 48. In certain embodiments, the NHBA polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 48.

[00194] In certain embodiments, the NHBA polypeptide is a C-terminal amino acid fragment of the full length NHBA polypeptide. In certain embodiments, the NHBA polypeptide is a C-terminal, 178-188 amino acid fragment of the full length NHBA polypeptide (*e.g.*, of SEQ ID NO: 15). In certain embodiments, the NHBA polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16. In certain embodiments, the NHBA polypeptide is designated as “cNHBA”.

[00195] In certain embodiments, the NHBA polypeptide disclosed herein is fused to a MetQ polypeptide to form a NHBA-MetQ fusion polypeptide. In certain embodiments, the N-terminus of the NHBA polypeptide disclosed herein is fused to the C-terminus of a MetQ polypeptide to form a NHBA-MetQ fusion polypeptide. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal

cysteine. In certain embodiments, the N-terminus of the MetQ polypeptide is fused to the C-terminus of the NHBA polypeptide to form a NHBA-MetQ fusion polypeptide.

[00196] In certain embodiments, the NHBA-MetQ fusion polypeptide further comprises a linker between the MetQ polypeptide and the NHBA polypeptide. In certain embodiments, the linker is a glycine-rich linker. In certain embodiments, the linker comprises the amino acid sequence GGGG (SEQ ID NO: 68).

[00197] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16.

[00198] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 17.

[00199]

[00200] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 57.

[00201] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 58.

[00202] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7.

[00203] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide comprises a MetQ signal peptide. In certain embodiments, the MetQ signal peptide comprises the amino acid sequence of SEQ ID NO: 6. In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the MetQ polypeptide lack all or a portion of the MetQ signal sequence, *i.e.*, truncated at the N-terminus. In certain embodiments, the MetQ polypeptide lacking the MetQ signal peptide disclosed herein comprises the amino acid sequence of SEQ ID NO: 19.

[00204] In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 20. In certain embodiments, the NHBA-MetQ fusion polypeptide provided herein comprises a MetQ signal peptide. In some embodiment, the MetQ signal peptide comprises the amino acid sequence of SEQ ID NO: 6.

[00205] In certain embodiments, the NHBA-MetQ fusion polypeptide disclosed herein further comprises a MetQ signal peptide. In certain embodiments, the MetQ signal peptide comprises the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the NHBA-MetQ fusion polypeptide lacks all or a portion of the MetQ signal sequence.

[00206] In certain embodiments, the MetQ signal peptide is removed during post-translational modifications. In certain embodiments, the NHBA-MetQ fusion polypeptide provided herein lack all or a portion of the MetQ signal sequence, *i.e.*, truncated at the N-terminus. In certain embodiments, the NHBA-MetQ fusion polypeptide provided herein comprises amino acids 19-199 corresponding to amino acids of SEQ ID NO: 20. In certain embodiments, the NHBA-MetQ fusion polypeptide lacking the MetQ signal peptide disclosed herein comprises the amino acid sequence set forth in SEQ ID NO: 21.

[00207] In certain embodiments of the NHBA-MetQ fusion polypeptide provided herein, the cysteine at the N-terminal of the NHBA-MetQ fusion polypeptide is lipidated. In certain embodiments, the signal peptide removal is coupled with the lipidation process. In certain embodiments, the signal peptide removal and the lipidation process are carried out by *E. coli* SEC-SPII pathway. In certain embodiments, the lipidation process is as well coupled with the export of the protein and results in the anchoring of the protein in *E. coli* outer membrane. An exemplary lipidation process is described in Nakayama et al., FEBS J (2012);279(23):4247-68, the content of which is incorporated by reference in its entirety. In certain embodiments, the anchoring of the NHBA-MetQ fusion polypeptide in *E. coli* outer membrane results in the incorporation of the NHBA-MetQ fusion polypeptide in the OMV outer membrane through the releasing of the OMV from the *E. coli*.

[00208] In certain embodiments of the NHBA-MetQ fusion polypeptide comprises amino acid substitutions or deletions, which do not change the immunogenic activity of the NHBA-MetQ fusion polypeptide. In certain embodiments of the NHBA-MetQ fusion comprises amino acid substitutions or deletions, which improve the immunogenic activity of the NHBA-MetQ fusion polypeptide. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NOs: 60-65 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant retains the immunogenicity of SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NOs: 60-65. In certain embodiments, one or more of the amino acid residues of SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NOs: 60-65 may be modified (*e.g.*, by amino acid substitution or deletion) so that the variant improves the immunogenicity of SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NOs: 60-65.

[00209] In certain embodiments of the NHBA-MetQ fusion polypeptide comprises the NHBA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at

least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 16, and the MetQ polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 17. In certain embodiments of the NHBA-MetQ fusion polypeptide comprises the NHBA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 16, and the MetQ polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 19.

[00210] In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 20. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 21.

[00211] In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 41. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 41.

[00212] In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO: 49. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 49.

[00213] In certain embodiments of the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 16, and a MetQ polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 57. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising the amino acid sequence of SEQ ID NO: 16 and a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 57. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of the MetQ polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 60. In certain embodiments, the N-terminus of the MetQ polypeptide is fused to the C-terminus of the NHBA polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 63. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 60. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 63.

[00214] In certain embodiments of the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 16, and a MetQ polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 58. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising the amino acid sequence of SEQ ID NO: 16 and a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 58. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of the MetQ polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 61. In certain embodiments, the N-terminus of the MetQ polypeptide is fused to the C-terminus of the NHBA polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 64. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 61. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 64.

[00215] In certain embodiments of the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 16, and a MetQ polypeptide comprising an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least

about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises a NHBA polypeptide comprising the amino acid sequence of SEQ ID NO: 16 and a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the N-terminus of the NHBA polypeptide is fused to the C-terminus of the MetQ polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 62. In certain embodiments, the N-terminus of the MetQ polypeptide is fused to the C-terminus of the NHBA polypeptide. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 65. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 62. In certain embodiments, the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 65.

5.1.3 Modified Lipid A

[00216] The presently disclosure also provides an OMV comprising a modified lipid A. In certain embodiments, the modified lipid A has the benefit of enhancing immune responses against the pathogen while minimizing toxicity of the OMV vaccine.

[00217] In certain embodiments, the modified lipid A is covalently linked to a glycan antigen (*e.g.*, a glycan antigen disclosed in Section 5.1.1). In certain embodiments, the modified lipid A is linked to the glycan antigen via a co-localization in an outer membrane of an *E. coli* that produces the OMV.

[00218] In certain embodiments, the *E. coli* that produces the OMV comprises genetic modifications to produce the modified lipid A (*e.g.* the genetic modifications disclosed in Section 5.2.3). In certain embodiments, the *E. coli* comprises at least one lipid A modification enzyme that produces the modified lipid A. In certain embodiments, the at least one lipid A modification enzyme is selected from the group consisting of msbB, pagL, lpxE, pagP, and combinations thereof. In certain embodiments, the msbB, pagL, and/or lpxE is encoded by at least one exogenous

polynucleotide of the *E. coli* that produces the OMV. In certain embodiments, the *E. coli* further comprises genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes. In certain embodiments, the at least one lipid A modification enzyme is selected from the group consisting of pagL, lpxE, pagP, and combinations thereof. In certain embodiments, the pagL and/or lpxE is encoded by at least one exogenous polynucleotide of the *E. coli* that produces the OMV. In certain embodiments, the *E. coli* further comprises genetic disruption of *eptA*, and/or *lpxT* genes. In certain embodiments, the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

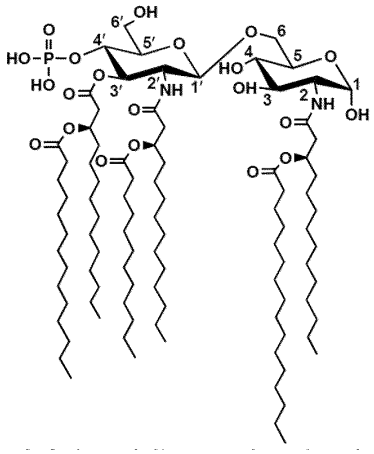
[00219] In certain embodiments, the modified lipid A is produced by a combination of msbB, pagL, lpxE, and pagP, the genetic disruption of *msbB*, *eptA*, and *lpxT* genes, and a lambda PL promoter that replaces the promoter of *pagP* gene, wherein the msbB, pagL, and lpxE are encoded by at least one exogenous polynucleotide of the *E. coli* that produces the OMV.

[00220] In certain embodiments, the genetic disruption and then reinsertion of the *msbB* gene is functionally equivalent to making no genetic modification of the *msbB* gene. Thus, in certain embodiments, no genetic disruption of the *msbB* gene or reinsertion of the exogenous *msbB* gene is made in the *E. coli*. In certain embodiments, the modified lipid A is produced by a combination of pagL, lpxE, and pagP, the genetic disruption of *eptA*, and *lpxT* genes, and a lambda PL promoter that replaces the promoter of *pagP* gene, wherein each of the pagL and lpxE is encoded by an exogenous polynucleotide of the *E. coli* that produces the OMV.

[00221] MPL (*i.e.*, monophosphorylated lipid A) as used herein refers to a heterogeneous mixture of lipid A species from *Salmonella minnesota* R595 that has been chemically detoxified by successive acid and base hydrolysis. Description of MPL can be found in Needham *et al.*, *Proc Natl Acad Sci U S A*, 2013;110(4):1464-1469; Wang *et al.*, *Front Immunol*, 2020;11:577823; Hagen *et al.*, *J Chromatogr A*. 1997;767(1-2):53-61, and the content of each of which is incorporated by reference herein. MPL produced by different methods might include different species and with different relative abundance. MPL® exclusively manufactured by GSK (*see* Wang *et al.*, *Front Immunol*, 2020;11:577823; Hagen *et al.*, *J Chromatogr A*. 1997;767(1-2):53-61; Ulrich *et al.*, *Pharm Biotechnol.*, 1995:6:495-524) is a licensed vaccine component, used as a standalone adjuvant in Bexsero, and is a component of AS04 adjuvant system (present in Cervarix and Fendrix) and AS01B adjuvant system (used in Shingrix). **FIG. 3C** shows the approximate percentage of the amount of each species of the lipid A in the MPL® of GSK. **FIG. 3D** shows the chemical structure of the wildtype *E coli.* lipid A.

[00222] 3-O-desacyl-4′-monophosphoryl lipid A as used herein refers to a particular chemical structure among the structures found in MPL, where the 3-O-desacyl-4′-monophosphoryl lipid A

species contain only one phosphate group at the 4' position and lacks an acyl chain at the 3 position. An exemplary chemical structure of a 3-O-desacyl-4'-monophosphoryl lipid A species is as follows:



[00223] In certain embodiments, the structure of modified lipid A produced by the engineered *E. coli* strains is heterogeneous. In certain embodiments, the modified lipid A produced by the engineered *E. coli* strains is a mixture of heterogeneous lipid A species having different chemical structures. In certain embodiments, the modified lipid A produced by the engineered *E. coli* strains have heterogeneous structures but some common features, such as every structure contains at least two C14-3OH acyl chains, and/or every structure contains one C12 acyl chain. Exemplary modified lipid A structures are provided in **FIG. 3A**. In certain embodiments, at least one structure of modified lipid A produced by the engineered *E. coli* strains contains a C16 chain.

[00224] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species comprising only one phosphate group. In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone. In certain embodiments, at least 60% (*e.g.*, about 60%, about 70%, about 80%, or about 90%) of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone. In certain embodiments, between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone. In certain embodiments, about 73% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone.

[00225] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone. In certain

embodiments, at least 60% (*e.g.*, about 60%, about 70%, about 80%, or about 90%) of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone. In certain embodiments, between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone. In certain embodiments, about 89% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone.

[00226] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A. In certain embodiments, at least 50% (*e.g.*, about 50%, about 60%, about 70%, about 80%, or about 90%) of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A. In certain embodiments, between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A. In certain embodiments, about 64% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A.

[00227] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species comprising at least three (e.g., three, or four) C14-3OH acyl chains. In certain embodiments, at least 60% (*e.g.*, about 60%, about 70%, about 80%, about 90%, about 95%, or about 99%) of the amount of the modified lipid A comprises at least three C14-3OH acyl chains. In certain embodiments, between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains. In certain embodiments, about 98% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains.

[00228] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone. In certain embodiments, at least 30% (*e.g.*, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, or about 90%) of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone. In certain embodiments, between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone. In certain embodiments, about 43% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone.

[00229] In certain embodiments, the modified lipid A disclosed herein comprises at least one lipid A species comprising a C12 acyl chain. In certain embodiments, at least 60% (*e.g.*, about 60%, about 70%, about 80%, about 90%, about 95%, or about 99%) of the amount of the modified lipid A comprises a C12 acyl chain. In certain embodiments, between 90% and 100% of the

amount of the modified lipid A comprises a C12 acyl chain. In certain embodiments, about 98% of the amount of the modified lipid A comprises a C12 acyl chain.

[00230] In certain embodiments, the modified lipid A disclosed herein comprises (i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone; (ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone; (iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains; (v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and (vi) at least one lipid A species comprising a C12 acyl chain.

[00231] In certain embodiments, (i) at least 60% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone, (ii) at least 60% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone, (iii) at least 50% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A, (iv) at least 60% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains, (v) at least 30% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone, and (vi) at least 60% of the amount of the modified lipid A comprises a C12 acyl chain.

[00232] In certain embodiments, (i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone, (ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone, (iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A, (iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains, (v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone, and (vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

[00233] Exemplary chemical structures of the species of the presently disclosed modified lipid A are provided in **FIG. 3A**. Approximate percentage of the amount of each species of an exemplary modified lipid A disclosed herein is provided in **FIG. 3B**.

[00234] In certain embodiments, the modified lipid A comprises different structures with potentially different TLR-4 binding activities and a safe profile. The modified lipid A disclosed herein has a superior ability in activating TLR-4 (*e.g.*, inducing proinflammatory cytokines) and at

the same time retaining a safe profile (*see* Example 21). For example, OMVs carrying the modified lipid A disclosed herein do not cause temperature increase in a subject receiving the OMVs.

[00235] In certain embodiments, the modified lipid A are analogous to the structure of MPL from Salmonella enterica Minnesota.

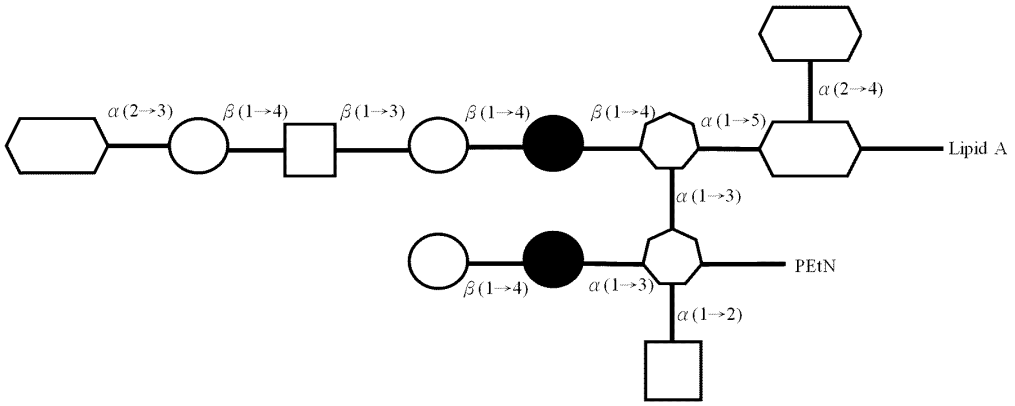
5.1.4 Exemplary OMVs

[00236] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising (i) protein antigens comprising an AniA polypeptide (*e.g.*, an AniA polypeptide disclosed in Section 5.1.2(a)), a MetQ polypeptide (*e.g.*, a MetQ polypeptide disclosed in Section 5.1.2(b)), a MsrA/B polypeptide (*e.g.*, a MsrA/B polypeptide disclosed in Section 5.1.2(c)), and a NHBA polypeptide (*e.g.*, a NHBA polypeptide disclosed in Section 5.1.2(d)); (ii) a glycan antigen comprising a KDO (*e.g.*, a KDO disclosed in Section 5.1.1(a)) and a 2C7 epitope (*e.g.*, a 2C7 epitope disclosed in Section 5.1.1(b)), and (iii) a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3), wherein the glycan antigen is covalently linked to the modified lipid A. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00237] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising (i) protein antigens comprising an AniA polypeptide (*e.g.*, an AniA polypeptide disclosed in Section 5.1.2(a)), a MetQ polypeptide (*e.g.*, a MetQ polypeptide disclosed in Section 5.1.2(b)), a MsrA/B-AniA fusion polypeptide (*e.g.*, a MsrA/B-AniA polypeptide disclosed in Section 5.1.2(c)), and a NHBA-MetQ fusion polypeptide (*e.g.*, a NHBA-MetQ fusion polypeptide disclosed in Section 5.1.2(d)); (ii) a glycan antigen comprising a KDO (*e.g.*, a KDO disclosed in Section 5.1.1(a)) and a 2C7 epitope (*e.g.*, a 2C7 epitope disclosed in Section 5.1.1(b)), and (iii) a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3), wherein the glycan antigen is covalently linked to the modified lipid A. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00238] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14, a MetQ

polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is covalently linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

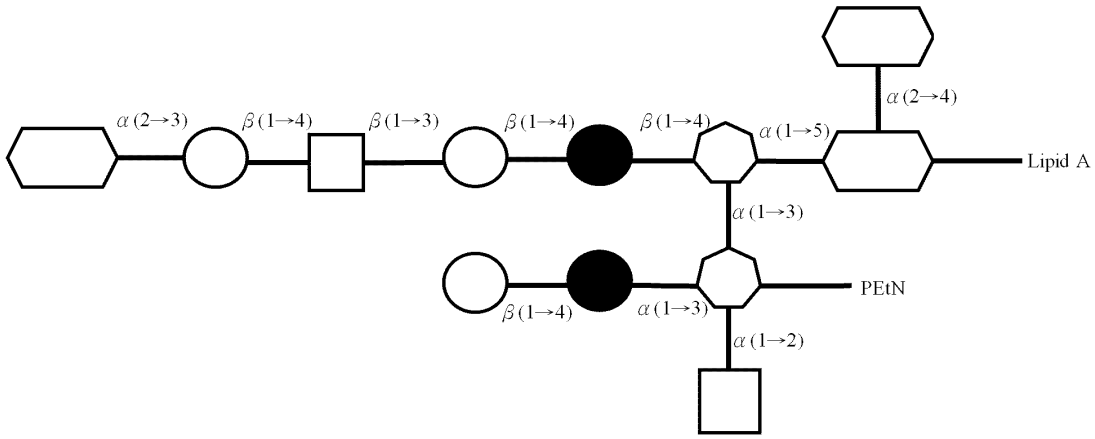


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of msbB, pagL, lpxE, and pagP, wherein the msbB, pagL, and lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00239] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 14, a MetQ polypeptide comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises an amino acid sequence having at

least 90% sequence identity to the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is covalently linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

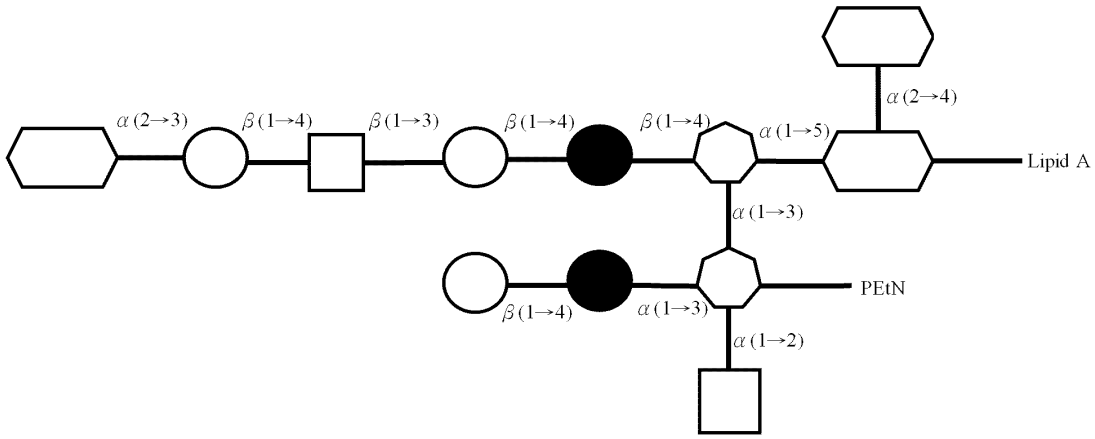


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of pagL, lpxE, and pagP, wherein each of the pagL and lpxE is encoded by an exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00240] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14, a MetQ polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4, and a

NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is covalently linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

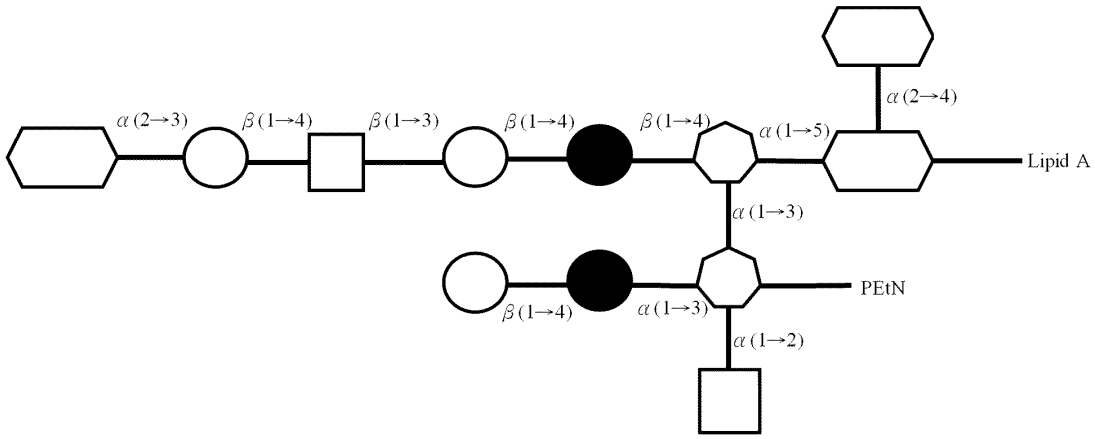


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A comprises (i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone; (ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone; (iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains; (v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and (vi) at least one lipid A species comprising a C12 acyl chain. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00241] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14, a MetQ

polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is covalently linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

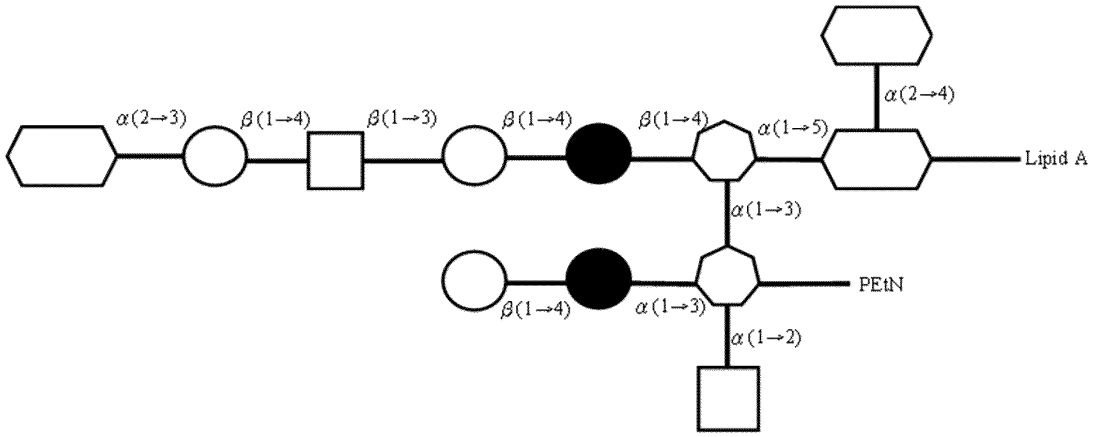


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine; and

wherein (i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone, (ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone, (iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A, (iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains, (v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone, and (vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but

are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00242] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14, a MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

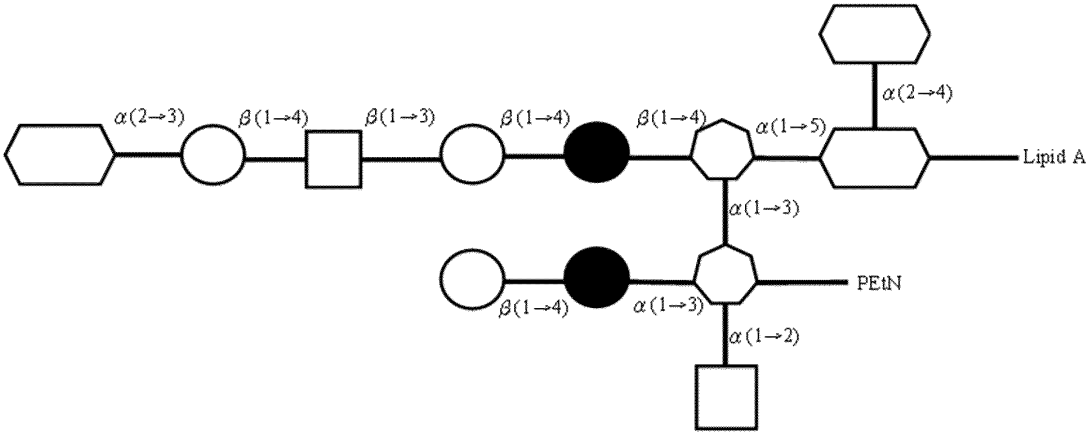


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of msbB, pagL, lpxE, and pagP, wherein the msbB, pagL, and lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00243] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises the amino acid sequence

of SEQ ID NO: 14, a MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the following structure and is linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

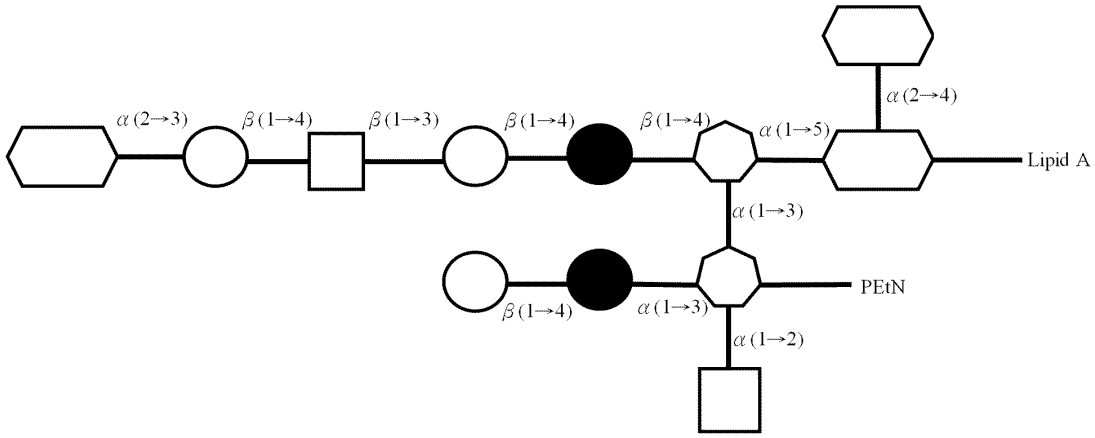


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of pagL, lpxE, and pagP, wherein each of the pagL and lpxE is encoded by an exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00244] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14, a MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the

following structure and is linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:

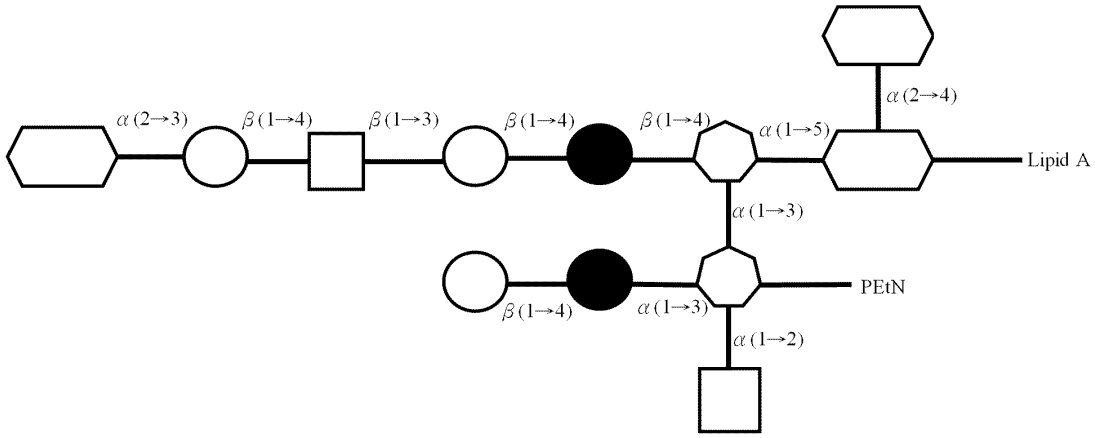


wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A comprises (i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone; (ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone; (iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains; (v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and (vi) at least one lipid A species comprising a C12 acyl chain. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00245] In certain embodiments, provided herein is an *E. coli*-derived OMV comprising: (i) protein antigens comprising a MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14, a MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7, an AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4, and a NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21, and (ii) a glycan antigen has the

following structure and is linked to a modified lipid A of the OMV as follows, and is shown in **FIG. 32B**:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine; and

wherein (i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone, (ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone, (iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A, (iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains, (v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone, and (vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain. In certain embodiments, the protein antigens and glycan antigen are associated with the outer membrane of the OMV and are exposed to the surface of the OMV. In certain embodiments, the protein antigens are associated with the outer membrane of the OMV but are not exposed to the surface of the OMV. In certain embodiments, the KDO can be recognized by antibody 6E4.

[00246] In certain embodiments, the expression of protein antigens on the OMVs are assessed, *e.g.*, by immunoblots or ELISAs. In certain embodiments, the expression of glycan antigens on the OMVs are assessed, *e.g.*, by immunoblots or ELISAs.

5.1.5 Additional Features of OMVs Derived From *E. coli*

[00247] OMVs disclosed herein are produced from *E. coli*, and thus are essentially free of any antigenic factors or protein antigens of *N. gonorrhoeae*, except the protein and glycan antigens disclosed herein (*e.g.*, protein antigens of Section 5.1.2, glycan antigens of Section 5.1.1). As such, OMVs disclosed herein do not elicit immune evasion and/or suppression mechanisms of *N. gonorrhoeae* that can reduce the protective efficacy of the OMV vaccine. In certain embodiments, the OMV does not comprise a *N. gonorrhoeae* protein selected from the group consisting of reduction modifiable protein (rmp), type IV pili protein (T4P), opacity-associated protein (Opa), HpuA, and HpuB of *N. gonorrhoeae*.

[00248] In certain embodiments, the proportions of specific glycolipids and /or phospholipids, including phosphatidylethanolamine, phosphatidylglycerol, and/or cardiolipin in the OMVs disclosed herein are different from OMVs produced from *N. gonorrhoeae*. In certain embodiments, OMVs disclosed herein have overall low to inexistent level of homology to the OMVs produced from *N. gonorrhoeae*.

[00249] In the outer membrane of *E. coli* and OMVs produced and derived thereof, the lipid A component, which is the hydrophobic anchor of lipopolysaccharide (LPS), constitutes almost 100% of the outer leaflet. As disclosed herein, in certain embodiments, the *E. coli*-derived OMVs disclosed herein comprise a modified lipid A (*e.g.*, modified lipid A disclosed in Section 5.1.3), whereas the lipid A of *N. gonorrhoeae*- derived OMVs has a different structure from the modified lipid A disclosed herein.

[00250] In certain embodiments, the present disclosure provides a composition comprising a plurality of OMVs disclosed herein. In certain embodiments, the purity of the OMVs in the composition is at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 99%, where the purity is calculated as the percentage of the area under the OMV containing peak compared to the area under the entire chromatogram as measured by SEC-HPLC. In certain embodiments, the purity of the OMVs in the composition is at least 99%, where the purity is calculated as the percentage of the area under the OMV containing peak compared to the area under the entire chromatogram as measured by SEC-HPLC. In certain embodiments, the OMVs in the composition do not aggregate as assessed by SEC-HPLC.

[00251] In certain embodiments, the composition comprising the plurality of OMVs disclosed herein is essentially free of DNA from *E coli.* In certain embodiments, the composition comprises less than 5 ng, less than 4 ng, less than 3 ng, less than 1 ng, less than 100 pg, less than 50 pg, less than 25 pg, less than 10 pg, less than 5 pg, or less than 1.5 pg, DNA from *E coli* /μg total protein as

measure by qPCR. In certain embodiments, the composition comprising the plurality of OMVs disclosed herein comprises less than 1.5 pg DNA from *E coli* /μg total protein as measure by qPCR.

[00252] In certain embodiments, the diameter of the OMVs in the presently disclosed composition is between 20 nm and 80 nm, between 30 nm and 70 nm, between 40 nm and 60 nm, or between 50 nm and 60 nm, as measured by DLS. In certain embodiments, the diameter of the OMV disclosed herein, as measured by DLS, is about 30 nm, about 35 nm, about 40 nm, about 45 mg (*e.g.*, 44.5 nm), about 50 nm, about 55 nm, or about 60 nm.

[00253] In certain embodiments, no significant degradation of OMVs in the presently disclosed composition is observed after being stored at 2°C - 8°C for at least about 4 week, at least about 8 week, at least about 12 week, at least about 16 week, at least about 20 week, at least about 24 week, at least about 28 week, at least about 32 week, at least about 36 week, at least about 40 week, at least about 44 week, at least about 48 week, at least about 52 week, at least about 56 week, at least about 1 year, or more.

5.2 Engineered *Escherichia Coli* for Producing OMVs

[00254] In certain embodiments, the present disclosure further provides an engineered *E. coli* for producing an OMV disclosed herein, wherein the *E. coli* comprises (i) at least one glycan antigen (*e.g.*, at least one glycan of Section 5.1.1), (ii) at least one protein antigen (*e.g.*, at least one protein agent of Section 5.1.2), and/or (iii) a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3). In certain embodiments, the at least one glycan antigen and/or the at least one protein antigen are antigens of a pathogen (*e.g.*, *N. gonorrhoeae*).

[00255] In certain embodiments, the *E. coli* comprises (i) one or more genetic modifications for producing the at least one glycan antigen (*e.g.*, one or more genetic modifications of Section 5.2.1); (ii) one or more genetic modifications for producing the at least one protein antigen (*e.g.*, one or more genetic modifications of Section 5.2.2); (iii) one or more genetic modifications for producing the modified lipid A (*e.g.*, one or more genetic modifications of Section 5.2.3); (iv) one or more additional genetic modifications (*e.g.*, one or more additional genetic modifications of Section 5.2.4). In certain embodiments, the *E. coli* produces an OMV as described herein (*e.g.*, OMVs of Section 5.1).

5.2.1 Genetic Modifications for Expressing Glycan Antigens

[00256] In certain embodiments, the present disclosure provides an engineered *E. coli* comprising at least one glycan antigen (*e.g.*, at least one glycan of Section 5.1.1). In certain embodiments, the at least one glycan antigen is associated with the outer membrane of the *E. coli*, for example, by covalently conjugated to a lipid A associated with the outer membrane. Under

suitable conditions, the *E. coli* produces and releases OMVs, where part of the outer membrane of the *E. coli* becomes the outer membrane of the OMVs, and thus the at least one glycan antigen associated with the outer membrane of the *E. coli* is incorporated into the OMVs. In certain embodiments, the *E. coli* comprises genetic modifications for generating the at least one glycan antigen. In certain embodiments, the *E. coli* produces an OMV comprising the at least one glycan antigen (*e.g.*, at least one glycan of Section 5.1.1).

[00257] In certain embodiments, the *E. coli* is derived from *E. coli* C600 strain.

[00258] In certain embodiments, the *E. coli* comprises one or more or all of the following genetic modifications to be able to biosynthesize at least one glycan antigen disclosed herein:

(i) clusters for the biosynthesis of *E. coli* K12 LPS core *waaQGPSBORYZU* (GenBank NZ_CP031214 position 117,878 to 127,774), which was replaced by an artificial operon containing genes for the biosynthesis of the LOS *lgtA* (*N. gonorrhoeae* 1291, GenBank ID AAF14358), *lgtB* (*N. mengitidis* MC58, GenBank ID AAF42257), *lgtE* (*N. gonorrhoeae* 1291, GenBank ID AAF14363), *lpt3* (*N. mengitidis* C311, GenBank ID QXZ29326), *lgtF* (*N. mengitidis* MC58, GenBank ID AAF42052), *rfaK* (*N. mengitidis* MC58, GenBank ID AAF42053), *lgtG* (*N. mengitidis* M22790, GenBank ID AOA42063), controlled by the existing genomic promoter;

(ii) the genomic locus *wza-wbbL* (GenBank NZ_CP031214 position 1,786,050 to 1,822,024), including the colanic acid cluster *wca*, *galF*, and the O antigen cluster *rfb*, which was replaced by *lst* from Neisseria gonorrhoeae 1291 (GenBank AAY41933) and further copies of *lgtB*, *lgtA* and *lgtE*, under the control of the constitutive promoter of *E. coli*'s *rfb* cluster which has been reinserted;

(iii) enterobacterial common antigen (ECA) cluster (*wecA – rffM*, GenBank NZ_CP031214 position 4,557,910 - 4,545,870) which was replaced by further copies of *lgtB* and lgtA;

(iv) an additional copy of the *lst* (SEQ ID NO: 37) from *Neisseria gonorrhoeae* (GenBank CP078119: 2,138,146 - 2,139,282) is introduced into a pBAD-derived plasmid under constitutive phage promoter;

(v) 3-deoxy-manno-octulosonate cytidylyltransferase *kdsB* (SEQ ID NO. 53) from *E. coli* (GenBank NZ_CP031214 position 2,949,898 to 2,950,644) is introduced on a pEXT20 expression plasmid (*e.g.*, pLTPro231 or pLTPro302) under an IPTG-inducible promoter or a J23105 promoter via standard cloning techniques.

[00259] In certain embodiments, the *E. coli* comprises genetic disruption of LOS gene cluster. In certain embodiments, the LOS gene cluster comprises *waa* genes. In certain embodiments, the LOS gene cluster comprises *waaQGPSBORYZU* (GenBank NZ_CP031214 position 117,878 to 127,774).

In certain embodiments, the *E. coli* comprises genetic disruption of *waaQ*, *waaG*, *waaP*, *waaS*, *waaB*, *waaO*, *waaR*, *waaZ*, *waaY,* and/or *waaU* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *waaQ*, *waaG*, *waaP*, *waaS*, *waaB*, *waaO*, *waaR*, *waaZ*, *waaY,* and *waaU* genes.

[00260] In certain embodiments, the *E. coli* comprises genetic disruption of the genomic locus *wza-wbbL* (GenBank NZ_CP031214 position 1,786,050 to 1,822,024), including the colanic acid cluster *wca*, *galF*, and the O antigen cluster *rfb*.

[00261] In certain embodiments, the *E. coli* comprises genetic disruption of colanic acid biosynthesis gene cluster. In certain embodiments, the *E. coli* comprises genetic disruption of *wca* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL,* and/or *wcaM* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL,* and *wcaM* genes.

[00262] In certain embodiments, the *E. coli* comprises genetic disruption of O antigen biosysthesis gene cluster. In certain embodiments, the *E. coli* comprises genetic disruption of *rfb* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes.

[00263] In certain embodiments, the *E. coli* comprises genetic disruption of *galF* gene which is located in between of colanic acid cluster (wca) and O antigen cluster (rfb).

[00264] In certain embodiments, the *E. coli* comprises genetic disruption of ECA (enterobacterial common antigen) gene cluster. In certain embodiments, the ECA cluster comprises *wecA – wecG*, (GenBank NZ_CP031214 position 4,557,910 - 4,545,870). In certain embodiments, the *E. coli* comprises genetic disruption of *wec* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes. Alternative names of *wec* genes are as follows: *wecA* is also known as *rfe*; *wecB* is also known as *rffE*; *wecC* is also known as *rffD*; *rmlB* is also known as *rffG*; *rmlA* is also known as *rffH*, *wecC* is also known as *rffC; wecW* is also known as *rffA; wecF* is also known as *rffT; wecG* is also known as *rffM*.

[00265] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB. In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, the exogenous polynucleotide encoding lgtE, the exogenous polynucleotide encoding lgtA, the exogenous polynucleotide encoding lgtB, the exogenous polynucleotide encoding lst are incorporated into the genome of the *E. coli*. In certain embodiments, the exogenous polynucleotide encoding lst is expressed from a vector. In certain embodiments, the expression of lst is constitutively under control of lambda phage promoter PL in the vector. In certain embodiments, the exogenous polynucleotide encoding kdsB is expressed from a vector.

[00266] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding lgtB, at least one exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, two exogenous polynucleotides each encoding lgtE, three exogenous polynucleotides each encoding lgtA, three exogenous polynucleotides each encoding lgtB, two exogenous polynucleotides each encoding lst, and/or an exogenous polynucleotide encoding kdsB.

[00267] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding

lgtB, at least one exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, two exogenous polynucleotides each encoding lgtE, three exogenous polynucleotides each encoding lgtA, three exogenous polynucleotides each encoding lgtB, two exogenous polynucleotides each encoding lst, and an exogenous polynucleotide encoding kdsB.

[00268] In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, one of the at least one exogenous polynucleotide encoding lst, the at least one exogenous polynucleotide encoding lgtE, the at least one exogenous polynucleotide encoding lgtA, and the at least one exogenous polynucleotide encoding lgtB are incorporated into the genome of the *E. coli*. In certain embodiments, one of the at least one exogenous polynucleotide encoding lst is expressed from a vector. In certain embodiments, the expression of lst is constitutively under control of lambda phage promoter PL in the vector. In certain embodiments, the exogenous polynucleotide encoding kdsB is expressed from a vector.

[00269] In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, one of the two exogenous polynucleotides encoding lst, the two exogenous polynucleotides encoding lgtE, the three exogenous polynucleotides encoding lgtA, and the three exogenous polynucleotides encoding lgtB are incorporated into the genome of the *E. coli*. In certain embodiments, one of the two exogenous polynucleotides encoding lst is expressed from a vector. In certain embodiments, the expression of lst is constitutively under control of lambda phage promoter PL in the vector. In certain embodiments, the exogenous polynucleotide encoding kdsB is expressed from a vector.

[00270] In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB; genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and/or *waaU* genes; genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI,*

wbbJ, wbbL', intS, and/or *'wbbL*; and/or genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes. In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB; genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes; genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes; and genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes.

[00271] In certain embodiments, the exogenous polynucleotides encoding lgtA, lgtB, lgtE, lpt3, lgtF, rfaK and/or lgtG are incorporated into the genome of the *E. coli*. In certain embodiments of the *E. coli* provided herein, *waa* genes (*waaQ-U* genes) has been replaced with exogenous polynucleotides encoding lgtA, lgtB, lgtE, lpt3, lgtF, rfaK and/or lgtG. In certain embodiments of the *E. coli* provided herein, *waa* genes (*waaQ-U* genes) has been replaced with exogenous polynucleotides encoding lgtA, lgtB, lgtE, lpt3, lgtF, rfaK and lgtG. In certain embodiments of the *E. coli* provided herein, *waa* genes (*waaQ-U* genes) has been replaced with exogenous polynucleotides encoding one copy of each of lgtA, lgtB, lgtE, lpt3, lgtF, rfaK and lgtG. *Waa* genes cluster include a set of genes involved in the synthesis, modification, and transport of LOS molecules. In certain embodiments, *waa* genes include *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes.

[00272] In certain embodiments, the exogenous polynucleotides encoding lst, lgtB, lgtA, and/or lgtE are incorporated into the genome of the *E. coli*. In certain embodiments of the *E. coli* provided herein, adjacent gene cluster for colanic acid and O antigen biosynthesis (*wca-rfb* genes) have been replaced with exogenous polynucleotides encoding lst, lgtB, lgtA, and/or lgtE. In certain embodiments of the *E. coli* provided herein, adjacent gene cluster for colanic acid and O antigen biosynthesis (*wca-rfb* genes) have been replaced with exogenous polynucleotides encoding lst, lgtB, lgtA, and lgtE. In certain embodiments of the *E. coli* provided herein, adjacent gene cluster for colanic acid and O antigen biosynthesis (*wca-rfb* genes) have been replaced with exogenous polynucleotides encoding one copy of each of lst, lgtB, lgtA, and lgtE. In certain embodiments, the constitutive promoter naturally driving the O antigen expression has been re-inserted to drive the

expression of exogenous polynucleotides encoding lst, lgtB, lgtA, and/or lgtE. An exemplary sequence comprises the sequence of O antigen promoter is provided as SEQ ID NO: 56. In certain embodiments, the constitutive promoter naturally driving the O antigen expression has been re-inserted to drive the expression of exogenous polynucleotides encoding lst, lgtB, lgtA, and lgtE. Colanic acid biosynthesis gene cluster include a set of genes involved in the synthesis of colanic acid. In certain embodiments, colanic acid biosynthesis gene cluster include *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL,* and *wcaM* genes. O antigen biosynthesis gene cluster include a set of genes involved in the biosynthesis and assembly of the O antigen component of lipopolysaccharides (LPS) or other surface polysaccharides. In certain embodiments, O antigen biosynthesis gene cluster include *rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *galF* gene which is located in between of colanic acid cluster (wca) and O antigen cluster (rfb). The replaced genes clusters have been selected as they might create interference in the glycan biosynthesis and accumulate unwanted glycans on the OMV.

[00273] In certain embodiments, the exogenous polynucleotides encoding lgtB and/or lgtA are incorporated into the genome of the *E. coli*. In certain embodiments of the *E. coli* provided herein, gene cluster for ECA (enterobacterial common antigen) (*wec* genes) has been replaced with exogenous polynucleotides encoding lgtB and/or lgtA. In certain embodiments of the *E. coli* provided herein, gene cluster for ECA (*wec* genes) has been replaced with exogenous polynucleotides encoding lgtB and lgtA. In certain embodiments of the *E. coli* provided herein, gene cluster for ECA (*wec* genes) has been replaced with exogenous polynucleotides encoding one copy of lgtB and one copy of lgtA. In certain embodiments, the natural ECA constitutive promoter drives the expression of exogenous polynucleotides encoding lgtB and/or lgtA. In certain embodiments, the natural ECA constitutive promoter drives the expression of exogenous polynucleotides encoding lgtB and lgtA. An exemplary sequence comprises the sequence of the ECA promoter is provided as SEQ ID NO: 55. Enterobacterial Common Antigen (ECA) gene cluster include a set of genes involved in the biosynthesis and assembly of the enterobacterial common antigen. In certain embodiments, ECA gene cluster include *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes. The replaced genes clusters have been selected as they might create interference in the glycan biosynthesis and accumulate unwanted glycans on the OMV.

[00274] In certain embodiments, the Lpt3 is a Lpt3 from *Neisseria meningitidis* C311, which has a GenBank number QXZ29326. In certain embodiments, the Lpt3 comprises the amino acid

sequence set forth in SEQ ID NO: 27. In certain embodiments, *lpt3* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lpt3* gene is inserted to replace *waaQ-U* genes.

[00275] In certain embodiments, the RfaK is a RfaK from *Neisseria meningitidis* MC58, which has a GenBank number AAF42053. In certain embodiments, the RfaK comprises the amino acid sequence set forth in SEQ ID NO: 28. In certain embodiments, *rfaK* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *rfaK* gene is inserted to replace *waaQ-U* genes.

[00276] In certain embodiments, the LgtF is a LgtF from *Neisseria meningitidis* MC58, which has a GenBank number AAF42052. In certain embodiments, the LgtF comprises the amino acid sequence set forth in SEQ ID NO: 29. In certain embodiments, *lgtF* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lgtF* gene is inserted to replace *waaQ-U* genes.

[00277] In certain embodiments, the LgtG is a LgtG from *Neisseria meningitidis* M22790, which has a GenBank number AOA42063. In certain embodiments, the LgtG comprises the amino acid sequence set forth in SEQ ID NO: 30. In certain embodiments, *lgtG* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lgtG* gene is inserted to replace *waaQ-U* genes.

[00278] In certain embodiments, the LgtE is a LgtE from *N. gonorrhoeae* FA19, which has a GenBank number AKP11657. In certain embodiments, the LgtE comprises the amino acid sequence set forth in SEQ ID NO: 31. In certain embodiments, *lgtE* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lgtE* gene is inserted to replace *waaQ-U* genes.

[00279] In certain embodiments, the LgtE is a LgtE from *N. gonorrhoeae* 1291, which has a GenBank number GenBank AAF14363. In certain embodiments, the LgtE comprises the amino acid sequence set forth in SEQ ID NO: 32. In certain embodiments, *lgtE* gene is inserted at *wca-rfb* gene locus. In certain embodiments, *lgtE* gene is inserted to replace *wca-rfb* genes.

[00280] In certain embodiments, the LgtA is a LgtA from *Neisseria meningitidis* MC58, which has a GenBank number AAF42258. In certain embodiments, the LgtA comprises the amino acid sequence set forth in SEQ ID NO: 33. In certain embodiments, *lgtA* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lgtA* gene is inserted to replace *waaQ-U* genes.

[00281] In certain embodiments, the LgtA is a LgtA from *N. gonorrhoeae 1291,* which has a GenBank number AAF14359. In certain embodiments, the LgtA comprises the amino acid sequence set forth in SEQ ID NO: 34. In certain embodiments, *lgtA* gene is inserted at *wca-rfb* gene locus. In certain embodiments, *lgtA* gene is inserted to replace *wca-rfb* genes. In certain embodiments, *lgtA* gene is inserted at *wec* gene locus. In certain embodiments, *lgtA* gene is inserted to replace *wec* genes.

[00282] In certain embodiments, the LgtB is a LgtB from *Neisseria meningitidis* MC58, which has a GenBank number AAF42257. In certain embodiments, the LgtB comprises the amino acid sequence set forth in SEQ ID NO: 35. In certain embodiments, *lgtB* gene is inserted at *waaQ-U* gene locus. In certain embodiments, *lgtB* gene is inserted to replace *waaQ-U* genes.

[00283] In certain embodiments, the LgtB is a LgtB from (*wca-rfb* locus and *wec* locus) from *N. gonorrhoeae 1291*, which has a GenBank number AAF14360. In certain embodiments, the LgtB comprises the amino acid sequence set forth in SEQ ID NO: 36. In certain embodiments, *lgtB* gene is inserted at *wca-rfb* gene locus. In certain embodiments, *lgtB* gene is inserted to replace *wca-rfb* genes. In certain embodiments, *lgtB* gene is inserted at *wec* gene locus. In certain embodiments, *lgtB* gene is inserted to replace *wec* genes.

[00284] In certain embodiments, the Lst is a Lst (pLTPro159 and *wca-rfb* locus) from *N. gonorrhoeae* 1291, which has a GenBank number AAY41933. In certain embodiments, the Lst comprises the amino acid sequence set forth in SEQ ID NO: 37. In certain embodiments, *lst* gene is inserted at *wca-rfb* gene locus. In certain embodiments, *lst* gene is inserted to replace *wca-rfb* genes.

[00285] In certain embodiments, a polynucleotide encoding at least one enzyme for producing a glycan antigen can be cloned into an expression vector suitable for the expression system of choice. In certain embodiments, a polynucleotide encoding at least one enzyme for producing a glycan antigen is operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00286] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding KdsB. In certain embodiments, the *E. coli* comprises an expression vector comprising the exogenous polynucleotide encoding KdsB. In certain embodiments, the expression vector is a plasmid. In certain embodiments, the backbone of the expression vector is pEXT20 (Dykxhoorn, et al. 1996). In certain embodiments, the expression vector is plasmid pLTPro231 disclosed herein. In certain embodiments, the expression vector is plasmid pLTPro302 disclosed herein. In certain embodiments, the exogenous polynucleotide encoding KdsB is under the control of an IPTG-inducible promoter. In certain embodiments, the exogenous polynucleotide encoding KdsB is under the control of a J23105 promoter. In certain embodiments, the KdsB is a KdsB from *Escherichia coli* K12, which has a GenBank number WP_000011603. In certain embodiments, the KdsB comprises the amino acid sequence set forth in SEQ ID NO: 53. In certain embodiments, the KdsB comprises the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 54.

[00287] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding Lst. In certain embodiments, the *E. coli* comprises an expression vector comprising the exogenous polynucleotide encoding Lst. In certain embodiments, the expression vector is a plasmid. In certain embodiments, the original backbone of the expression vector is pBAD vector. In certain embodiments, the polynucleotide encoding Lst is under a lambda phage promoter PL. In certain embodiments, Lst is expressed constitutively under control of lambda phage promoter PL. In certain embodiments, the Lst is a Lst from *N. gonorrhoeae* 1291, which has a GenBank number AAY41933. In certain embodiments, the Lst comprises the amino acid sequence set forth in SEQ ID NO: 37.

[00288] In certain embodiments of the *E. coli* provided herein, the genomic manipulations are achieved homologous recombination techniques. Exemplary technology used for homologous recombination are described in Datsenko et al. Proc Natl Acad Sci U S A. (2000) 6;97(12):6640-5, the content of which is incorporated by reference in its entirety.

5.2.2 Genetic Modifications for Expressing Protein Antigens

[00289] In certain embodiments, the present disclosure further provides an engineered *E. coli* comprising at least one protein antigen (*e.g.*, at least one protein antigen of Section 5.1.2), wherein the at least one protein antigen are antigens of a pathogen (*e.g.*, *N. gonorrhoeae*). In certain embodiments, the *E. coli* comprises genetic modifications for expressing the at least one protein antigen. In certain embodiments, the *E. coli* comprises an expression system (*e.g.*, vector or vectors, plasmid or plasmids) for expressing the at least one protein antigen. In certain embodiments, the protein antigen produced by the *E. coli* is located to and associated with the outer membrane of the *E. coli*, for example, through lipidated N-terminal cysteine. Under suitable conditions, the *E. coli* produces and releases OMVs, where part of the outer membrane of the *E. coli* becomes the outer membrane of the OMVs, and thus the protein antigen associated with the outer membrane of the *E. coli* is incorporated into the OMVs.

[00290] In certain embodiments, the *E. coli* produces OMVs as described herein (*e.g.*, OMVs of Section 5.1). In certain embodiments, the *E. coli* produces OMVs as described in Section 5.1. In certain embodiments, the *E. coli* provided herein produces OMVs comprising at least one protein antigen (*e.g.*, at least one protein antigen of Section 5.1.2).

[00291] In certain embodiments, the original *E. coli* strain is *E. coli* C600.

[00292] In certain embodiments, the *E. coli* provided herein comprises genetic modifications to express the at least one protein antigen (*e.g.*, at least one protein antigen of Section 5.1.2).

[00293] In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide; an exogenous polynucleotide encoding an AniA polypeptide; and/or an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide. In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide; an exogenous polynucleotide encoding an AniA polypeptide; and an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide.

[00294] In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and a signal polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide and a signal polypeptide; an exogenous polynucleotide encoding an AniA polypeptide and a signal polypeptide; and/or an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and a signal polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide and a signal polypeptide; an exogenous polynucleotide encoding an AniA polypeptide and a signal polypeptide; and an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a signal polypeptide.

[00295] In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide and a MetQ signal polypeptide; an exogenous polynucleotide encoding an AniA polypeptide and an AniA signal polypeptide; and/or an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide; an exogenous polynucleotide encoding a MetQ polypeptide and a MetQ signal polypeptide; an exogenous polynucleotide encoding an AniA polypeptide and an AniA signal polypeptide; and an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide.

[00296] In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3; an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid

sequence of SEQ ID NO: 7 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6; an exogenous polynucleotide encoding an AniA polypeptide comprising the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3; and/or an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the *E. coli* provided herein comprises: an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3; an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6; an exogenous polynucleotide encoding an AniA polypeptide comprising the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3; and an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6.

(a) Expression System and Vectors for Expressing AniA Antigens

[00297] In certain embodiments, the *E. coli* provided herein comprises genetic modifications for producing an AniA polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide comprising the amino acid sequence of SEQ ID NO: 4.

[00298] In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide and a signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide and an AniA signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide comprising the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding an AniA polypeptide comprising the amino acid sequence of SEQ ID NO: 2.

[00299] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the AniA polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic

acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00300] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the AniA polypeptide and the AniA signal polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00301] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the AniA polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments, the original backbone of the expression vector used herein is pEXT20 (Dykxhoorn, et al. 1996). In certain embodiments, the expression vector is plasmid pLTPro231 disclosed herein. In certain embodiments, the expression vector is plasmid pLTPro302 disclosed herein. In certain embodiments, the polynucleotide encoding the AniA polypeptide and the AniA signal polypeptide is under an IPTG-inducible promoter. In certain embodiments, the polynucleotide encoding the AniA polypeptide and the AniA signal polypeptide is under a J23105 promoter. In certain embodiments, the nucleotide sequence encoding the AniA polypeptide and the AniA signal polypeptide comprises the nucleotide sequence as set forth in SEQ ID NO: 38.

(b) Expression System and Vectors for Expressing MetQ Antigens

[00302] In certain embodiments, the *E. coli* provided herein comprises genetic modifications for producing a MetQ polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 57. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 58.

[00303] In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide and a signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide and a MetQ signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MetQ polypeptide comprising the amino acid sequence of SEQ ID NO: 5.

[00304] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MetQ polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00305] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00306] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MetQ polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments, the original backbone of the expression vector used herein is pEXT20 (Dykxhoorn, et al. 1996). In certain embodiments, the expression vector is plasmid pLTPro231 disclosed herein. In certain embodiments, the expression vector is plasmid pLTPro302 disclosed herein. In certain embodiments, the polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide is under an IPTG-inducible promoter. In certain embodiments, the polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide is under a J23105 promoter. In certain embodiments, the nucleotide sequence encoding the MetQ polypeptide and the MetQ signal polypeptide comprises the nucleotide sequence as set forth in SEQ ID NO: 39.

(c) Expression System and Vectors for Expressing MsrA/B-AniA fusion polypeptide

[00307] In certain embodiments, the *E. coli* provided herein comprises genetic modifications for producing a MsrA/B-AniA fusion polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 14.

[00308] In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and a signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide comprising the amino acid sequence of SEQ ID NO: 3. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 13.

[00309] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MsrA/B-AniA fusion polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00310] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00311] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the MsrA/B-AniA fusion polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments, the original backbone of the expression vector used herein is pEXT20 (Dykxhoorn, et al. 1996). In certain embodiments,

the expression vector is plasmid pLTPro231 disclosed herein. In certain embodiments, the expression vector is plasmid pLTPro302 disclosed herein. In certain embodiments, the polynucleotide encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide is under an IPTG-inducible promoter. In certain embodiments, the polynucleotide encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide is under a J23105 promoter. In certain embodiments, the nucleotide sequence encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide comprises the nucleotide sequence as set forth in SEQ ID NO: 40.

(d) Expression System and Vectors for Expressing NHBA-MetQ fusion polypeptide

[00312] In certain embodiments, the *E. coli* provided herein comprises genetic modifications for producing a NHBA-MetQ fusion polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 21.

[00313] In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide comprising the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 20. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 60. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 61. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 62. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 63. In certain embodiments, the *E. coli* provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 64. In certain embodiments, the

E. coli provided herein comprises an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide comprising the amino acid sequence of SEQ ID NO: 65.

[00314] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the NHBA-MetQ fusion polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00315] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide can be cloned into an expression vector suitable for the expression system of choice, operably linked to regulatory sequences that facilitate expression of the heterologous nucleic acid molecule. In general, expression vectors can include transcriptional promoters and optionally enhancers to which the polynucleotide is operably linked, translational signals, and transcriptional and translational termination signals.

[00316] In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the NHBA-MetQ fusion polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments of the *E. coli* provided herein, a polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide is cloned into an expression vector, wherein the expression vector is a plasmid. In certain embodiments, the original backbone of the expression vector used herein is pEXT20 (Dykxhoorn, et al. 1996). In certain embodiments, the expression vector is plasmid pLTPro231 disclosed herein. In certain embodiments, the expression vector is plasmid pLTPro302 disclosed herein. In certain embodiments, the polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide is under an IPTG-inducible promoter. In certain embodiments, the polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide is under a J23105 promoter. In certain embodiments, the nucleotide sequence encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide comprises the nucleotide sequence as set forth in SEQ ID NO: 41.

5.2.3 Genetic Modifications for Producing Modified Lipid A

[00317] In certain embodiments, the present disclosure further provides an engineered *E. coli* comprising a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3). In certain embodiments, the *E. coli* comprises genetic modifications for generating the modified lipid A. In certain embodiments, the *E. coli* comprises an expression system (*e.g.*, vector or vectors) for expressing at least one enzymes for generating the modified lipid A. In certain embodiments, the *E. coli* provided

herein produces OMVs as described in Section 5.1. In certain embodiments, the *E. coli* provided herein produces OMVs comprising a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3).

[00318] In certain embodiments, the original *E. coli* strain is *E. coli* C600.

[00319] In certain embodiments, the *E. coli* provided herein comprises genetic modifications to produce a modified lipid A (*e.g.*, a modified lipid A of Section 5.1.3).

[00320] In certain embodiments, the genetic modifications to produce a modified lipid A comprises one or more, or all of the following:

(i) lipid A 1-diphosphate synthase *lpxT* (GenBank NZ_CP031214 position 1,653,666 - 1,652,952) which was replaced by the lipid A 1-phosphatse *lpxE* from Francisella tularensis subsp novicida U112 (GenBank CP009633.1 position 1715820 - 1715101) preceded by the constitutive lambda PL promoter;

(ii) the promoter of the lipid A palmitoyltransferase *pagP* (GenBank NZ_CP031214 position 3,264,939 - 3,264,378) which was replaced by the constitutive lambda PL promoter;

(iii) the gene coding for the phosphoethanolamine transferase EptA (*eptA*, GenBank NZ_CP031214 position 1,982,323 - 1,983,295) which was replaced by the gene for lipid A 3-O-desacylase PagL from Salmonella enterica (*pagL*, GenBank CP123648.1, position 1710552 – 1711114) preceded by the lambda PL constitutive promoter (REF).

[00321] In certain embodiments, the engineered *E. coli* comprises: an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and/or an exogenous polynucleotide encoding lpxE. In certain embodiments, the engineered *E. coli* comprises: an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and an exogenous polynucleotide encoding lpxE. In certain embodiments, the exogenous polynucleotide encoding msbB, the exogenous polynucleotide encoding pagL, and the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*. In certain embodiments, the engineered *E. coli* comprises: an exogenous polynucleotide encoding pagL and/or an exogenous polynucleotide encoding lpxE. In certain embodiments, the engineered *E. coli* comprises an exogenous polynucleotide encoding pagL and an exogenous polynucleotide encoding lpxE. In certain embodiments, the exogenous polynucleotide encoding pagL and the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*.

[00322] In certain embodiments, the engineered *E. coli* comprises genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes. In certain embodiments, the engineered *E. coli* comprises genetic disruption of *msbB*, *eptA*, and *lpxT* genes. In certain embodiments, the engineered *E. coli* comprises

genetic disruption of *eptA* and/or *lpxT* genes. In certain embodiments, the engineered *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes.

[00323] In certain embodiments, the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[00324] In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and/or an exogenous polynucleotide encoding lpxE; genetic disruption of *msbB, eptA,* and/or *lpxT* genes; and the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[00325] In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and an exogenous polynucleotide encoding lpxE; genetic disruption of *msbB*, *eptA*, and *lpxT* genes; and the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[00326] In certain embodiments, the *msbB* gene is genetically disrupted (*e.g.*, removed) as the initial step, and the *msbB* gene is re-inserted in more advanced engineered *E. coli* strains. In certain embodiments, the initial genetic disruption of *msbB* renders the lipid A inactive in binding TLR4. In certain embodiments, *msbB* is re-inserted in a more advanced *E. coli* strain, wherein the integrated msbB is identical to the one originally removed. In certain embodiments, the MsbB is a MsbB from *E. coli* C600, which has a GenBank number WP_000448381. In certain embodiments, the MsbB comprises the amino acid sequence set forth in SEQ ID NO: 22.

[00327] In certain embodiments, the genetic disruption and then reinsertion of the *msbB* gene is functionally equivalent to making no genetic modification of the *msbB* gene. Thus, in certain embodiments, no genetic disruption of the *msbB* gene or insertion of an exogenous polynucleotide encoding msbB is made in the *E. coli*.

[00328] In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding pagL and/or an exogenous polynucleotide encoding lpxE; genetic disruption of *eptA* and/or *lpxT* genes; and the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding pagL and an exogenous polynucleotide encoding lpxE; genetic disruption of *eptA* and *lpxT* genes; and the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

[00329] In certain embodiments, the *pagL* gene is inserted to replace the *eptA* gene in the engineered *E. coli* strains. In certain embodiments, pagL is necessary for the removal of the C12-OH acyl chain from position 3 of the lipid A. In certain embodiments, the *pagL* gene is inserted under constitutive promoter control. In certain embodiments, the *eptA* gene is removed to prevent

its activity in transferring a phopsohoethanolamine on the position 1 of the lipid A. In certain embodiments, the PagL is a PagL from Salmonella enterica ssp. enterica serovar Typhimurium 14028S, which has a GenBank number WP_000281877. In certain embodiments, the MsbB comprises the amino acid sequence set forth in SEQ ID NO: 23.

[00330] In certain embodiments, the *lpxE* gene is inserted to replace the *lpxT* gene in the engineered *E. coli* strains. In certain embodiments, lpxE is necessary for the removal of the phosphate in position 1 of the lipid A. In certain embodiments, the *lpxE* gene is inserted under constitutive promoter control. In certain embodiments, the *lpxT* gene is removed to prevent its activity in adding a further unwanted phosphate group. In certain embodiments, the LpxE is a LpxE from Francisella tularensis ssp. Novicida U112, which has a GenBank number AAU11503. In certain embodiments, the LpxE comprises the amino acid sequence set forth in SEQ ID NO: 24.

[00331] In certain embodiments, the weak and regulated promoter of *pagL* gene is replaced by the lambda PL promoter. In certain embodiments, the constitutive pagL activity is necessary to add a C16 acyl chain to the C3 of the C14-OH acyl chain in position 2 of the lipid A. An exemplary PagP promoter sequence before substitution with lambda PL promoter is provided below:

[00332] PagP promoter before substitution with lambda PL promoter (start of PagP ORF underlined):

cgcatcatctttaatcgatgcgcggaaatatttaacttgaacaagcggaaataaatagagca
gctattcagattattctttatgttgggtctattaaggttatgttaattgtagctttgctatg
ctagtagtagatttttgataaatgttttatggtcacaaatgaacgtg (SEQ ID NO:
25)

[00333] An exemplary PagP promoter sequence after substitution with lambda PL promoter is provided below:

[00334] After substitution with lambda PL promoter (PL promoter bolded, FRT site double underlined, start of PagP ORF underlined):

cgcatcatctttaatcgatgcgatcgcccgcgaagttcctattccgaagttcctattctatc
agaagtataggaacttccggccggccttctttga**ttatctctggcggtgttgacataaatac**
cactggcggtgatactgagcacatcagcaggacgcactgaccgtaattatatttataaggag
gtaattctcgagatgaacgtg (SEQ ID NO: 26)

[00335] In certain embodiments of the *E. coli* provided herein, the genomic manipulations are achieved homologous recombination techniques. Exemplary technology used for homologous recombination are described in Datsenko et al. 2000. Proc Natl Acad Sci U S A. (2000) 6;97(12):6640-5, the content of which is incorporated by reference in its entirety.

5.2.4 Additional Genetic Modifications

[00336] In certain embodiments, the *E. coli* provided herein comprises genetic modifications to increase vesiculation.

[00337] In certain embodiments, the *E. coli* comprises: genetic disruption of *ompA* gene; genetic disruption of *lpp* gene; and/or an exogenous a polynucleotide encoding lpp. In certain embodiments, the *E. coli* comprises: genetic disruption of *ompA* gene; genetic disruption of *lpp* gene; and an exogenous polynucleotide encoding lpp. In certain embodiments, the *lpp* gene has GenBank NZ_CP031214 position 2,164,860 to 2,165,096.

[00338] In certain embodiments, the *E. coli* further comprises genetic disruption of *ompA* gene. OmpA is an integral outer membrane porin, which possesses a periplasmic domain which is covalently bound to the peptidoglycan, therefore stabilizing the outer membrane. In certain embodiments, *ompA* gene genetic disruption results in increased instability of the outer membrane, which therefore is released in form of OMVs. Exemplary approach has been employed in OMV vaccine described in Shigella GMMA. In certain embodiments, the *ompA* gene has GenBank NZ_CP031214 position 2,901,443 to 2,902,483.

[00339] In certain embodiments, the *E. coli* further comprises *lpp* gene genetic disruption and *lpp* gene re-insertion under arabinose-inducible promoter in the attλ locus of *E. coli*. Lpp is a heavily expressed small lipoprotein whose function is primarily to link the peptidoglycan to the outer membrane, to stabilize the latter. In certain embodiments, *lpp* gene genetic disruption results in de-stabilization of the outer membrane which is released as OMVs. In certain embodiments, *lpp* gene re-insertion is used as a form of arabinose-inducible OMV repression and/or OMV induction by arabinose depletion. The lpp re-insertion method used was described by Haldimann and Wanner, 2001, J Bacteriol. 2001 Nov;183(21):6384-93, the content of which is incorporated herein by reference in its entirety. In certain embodiments, the lpp expression is driven by a pBAD promoter. In certain embodiments, the *E. coli* further comprises an exogenous polynucleotide encoding lpp.

[00340] In certain embodiments, the genome of the *E. coli* comprises the nucleotide sequence set forth in SEQ ID NO: 42 as shown below, where attλ sites (duplicated upon insertion) are indicated by bold letters, polynucleotide encoding lpp is indicated by underlined letters; pBAD promoter is indicated by double underlined letters, FRT site is indicated by italic bold letters.

cagcaccgccgaaaggcgaaactcatcgctacatttttaccgttcacgcgctggatatagaac
gtattgatgtcgatgaaggtgccagcggcgcgatggtcgggtttaacgttcatttccactctc
tggcaagcgcctcgattactgcgatgtttagttaatcactctgccagatggcgcaatgccatc
tggtatcacttaaaggtattaaaaacaacttttttgtctttttaccttcccgtttcgctcaag**t**
tagtataaaaaagctgaacgagaaacgtaaaatgatataaatatcaatatattaaattagatt

ttgcataaaaaacagactacataatactgtaaaacacaacatatatgcagtcactatgaatca
actacttagatggtattagtgacctgtaacagagcattagcgcaaggtgatttttgtcttctt
gcgctaatttttttgtcatcaaacctgtcgcactccagagaagcacaaagcctcgcaatccagt
gcaaagctagcttcttcgtctgtttctactggtattggcacaaacctgattccaatttgagca
aggctatgtgccatctcgatactcgttcttaactcaacagaagatgctttgtgcatacagccc
ctcgtttattatttatctcctcagccagccgctgtgctttcagtggatttcggataacagaaa
ggccgggaaatacccagcctcgctttgtaacggagtagagacgaaagtgattgcgcctacccg
gatattatcgtgaggatgcgtcatcgccattaattcactgatcagtgataagctgtcaaacat
gagaattcgagctcggtacccgggttacttgcggtatttagtagccatgttgtccagacgctg
gttagcacgagctgcgtcatctttagcagcctgaacgtcggaacgcattgcgttcacgtcgtt
gctcagctggtcaactttagcgttcagagtctgaacgtcagaagacagctgatcgattttagc
gttgctggagcaacctgccagcagagtagaacccaggattaccgcgcccagtaccagtttagt
agctttcatatgaattcctccatccaaaaaaacgggtatggagaaacagtagagagttgcgat
aaaaagcgtcaggtaggatccgctaatcttatggataaaaatgctatggcatagcaaagtgtg
acgccgtgcaaataatcaatgtggacttttctgccgtgattatagacactttgctacgcgtt
tttgtcatggccttggtcccgctttgttacagaatgcttttaataagcggggttaccggtttg
gttagcgagaagagccagtaaaagacgcagtgacggcaatgtctgatgcaatatggacaattg
gtttcttctctgaatggcggctgcaggcatgcaagcttggcactggccacgcaaaaaggccat
ccgtcaggatggccttctgcttaatttgatgcctggcagtttatggcgggcgtcctgcccgcc
accctccgggccgttgcttcgcaacgttcaaatccgctcccggcggatttgtcctactcagga
gagcgttcaccgacaaacaacagataaaacgaaaggcccagtctttcgactgagcctttcgtt
ttatttgatgcctggcagttccctactctcgcatggggagaccccacactaccatcgggggcc
atcgatgcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaa
atacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatctagt
ggatcaagagacaggatgaggatcgtttcgcacatatgaatatcctcctt***agttcctattccg***
aagttcctattctctagaaagtataggaacttcgaagcagctccagcctacaccgggactctg
gggttcgataccgtcaaaacttcatttttaatttttgcggccgcaagatccggccacgatgcg
tccggcgtagaggatctgaagatcagcagttcaacctgttgatagtacgtactaagctctcat
gtttcacgtactaagctctcatgtttaacgtactaagctctcatgtttaacgaactaaaccct
catggctaacgtactaagctctcatggctaacgtactaagctctcatgtttcacgtactaagc
tctcatgtttgaacaataaaattaatataaatcagcaacttaaatagcctctaaggttttaag
ttttataagaaaaaaaagaatatataaggcttttaaagcttttaaggtttaacggttgtggac
aacaagccagggatgtaacgcactgagaagcccttagagcctctcaaagcaattttgagtgac
acaggaacacttaacggctgacatgggaattagccatggcatcacagtatcgtgatgacagag
gcagggagtgggacaaaattgaaatcaaataatgattttattttgactgatagtgacctgttc
gttgcaacaaattgataagcaatgctttttataatgccaac**ttagtataaaaaagc**aggctt
caacggattcatttttctatttcatagcccggagcaacctgtgaacacattttcagtttcccg
tctggcgctggcattggcttttggcgtgacgctgaccgcctgtagctcaaccccgcccgatca
acgtccttctgatcaaaccgcgcctggtacctcttctcgcccgatt (SEQ ID NO: 42)

[00341] In certain embodiment, the Lpp comprises the amino acid sequence set forth in SEQ ID NO: 43.

[00342] In certain embodiments, the *E. coli* provided herein comprises genetic disruption of *fliC* gene (GenBank NZ_CP031214, position 1,786,050 - 1,808,792). *FliC* gene encodes for flagellin, the structural protein of *E. coli*'s flagellum. In certain embodiments, the *fliC* gene genetic disruption is carried out to avoid any potential unwanted reactogenicity.

[00343] In certain embodiments, the *E. coli* provided herein comprises genetic disruption of *fim* gene cluster. *Fim* gene cluster is responsible for fimbriae biosynthesis. In certain embodiments, the *fim* gene cluster comprises *fimHGFDCIA* genes, GenBank NZ_CP031214, position 3,975,452 – 9,983,143. In certain embodiments, the *fim* gene cluster genetic disruption is carried out to avoid any potential unwanted reactogenicity.

[00344] In certain embodiments of the *E. coli* provided herein, the genomic manipulations are achieved homologous recombination techniques. Exemplary technology used for homologous recombination are described in Datsenko et al. 2000. Proc Natl Acad Sci U S A. (2000) 6;97(12):6640-5, the content of which is incorporated by reference in its entirety.

5.2.5 Vectors and Expression Systems

[00345] Some examples of vectors suitable for the disclosure may be a plasmid. Useful plasmids may include, but are not limited to, any plasmids described herein and capable of carrying and encoding the polypeptide as described herein. Some examples of viral vectors suitable for the disclosure include retrovirus -based vectors, *e.g.*, lentiviruses, adenoviruses, adeno- associated viruses (AAV), and vaccinia vectors. In certain embodiments, the structure of the vector may be modified as necessary for optimization of expression or to achieve a desired cellular level, of the polypeptide as described herein, such as including expression controlling elements (*e.g.*, promoter or enhancer sequences).

[00346] Polynucleotides useful in the present disclosure can be provided in an expression system. Expression systems of the disclosure generally include regulatory elements that are functional in the intended host cell (*e.g.*, *E. coli*) in which the expression system is to be expressed. Regulatory elements used for expression of nuclear genes include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements.

[00347] An expression system of the disclosure can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a polypeptide of the disclosure. As used herein, the term “operably linked” refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression construct of the disclosure. In some embodiment, a promoter can be positioned about the same distance from the transcription start site in the expression construct as it is from the transcription start site in its natural genetic environment. Some

variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

[00348] Nuclear expression constructs of the disclosure may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide, and/or enhancer elements. Transcription termination regions can typically be obtained from the 3 ' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. A signal peptide sequence is a short amino acid sequence typically present at the amino terminus of a protein that is responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of an operably linked signal peptide sequence is contemplated for use with the polypeptides of the disclosure. Classical enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Classical enhancer elements are known in the art, and include, but are not limited to, the cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. Intron-mediated enhancer elements that enhance gene expression are also known in the art. These elements must be present within the transcribed region and are orientation dependent.

[00349] Polynucleotides of the present disclosure can be composed of either RNA or DNA, or hybrids thereof. The present disclosure also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein. Polynucleotides and polypeptides of the disclosure can be provided in purified or isolated form.

[00350] In certain embodiments, such a promoter useful in accordance with the present disclosure may include any promoter sequences set forth herein, or other promoter sequences known and/or available in the art. In certain embodiments, primers useful for construction of a plasmid or vector as described herein may include any primer described herein. One of skill in the art will understand that other primers, plasmids, or vectors may be used without deviation from the scope of the present disclosure.

5.2.6 Exemplary Engineered *Escherichia Coli* for Producing OMVs

[00351] In certain embodiments, a *E. coli* provided herein for producing an OMV (*e.g.*, an OMV of Section 5.1) comprises (i) one or more genetic modifications for producing at least one glycan antigen (*e.g.*, one or more genetic modifications of Section 5.2.1); (ii) one or more genetic modifications for producing at least one protein antigen (*e.g.*, one or more genetic modifications of

Section 5.2.2); (iii) one or more genetic modifications for producing modified lipid A (*e.g.*, one or more genetic modifications of Section 5.2.3); and/or (iv) one or more additional genetic modifications (*e.g.*, one or more genetic modifications of Section 5.2.4).

[00352] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce at least one glycan antigen (*e.g.*, genetic modifications of Section 5.2.1). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises genetic disruption of *waa*Q, *waa*G, *waa*P, *waa*S, *waa*B, *waa*O, *waa*R, *waaY, waaZ*, and/or *waa*U genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes. In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, the exogenous polynucleotide encoding lgtE, the exogenous polynucleotide encoding lgtA, the exogenous polynucleotide encoding lgtB, and/or the exogenous polynucleotide encoding lst are incorporated into the genome of the *E. coli*. In certain embodiments, the exogenous polynucleotide encoding KdsB is expressed from a vector. In certain embodiments, the exogenous polynucleotide encoding Lst is expressed from a vector.

[00353] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce at least one glycan antigen (*e.g.*, genetic modifications of Section 5.2.1). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding lgtB, at least one exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, two exogenous polynucleotides each encoding lgtE, three

exogenous polynucleotides each encoding lgtA, three exogenous polynucleotides each encoding lgtB, two exogenous polynucleotides each encoding lst, and/or an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises genetic disruption of *waa*Q, *waa*G, *waa*P, *waa*S, *waa*B, *waa*O, *waa*R, *waaY, waaZ,* and/or *waa*U genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes. In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, one of the two exogenous polynucleotides encoding lst, the two exogenous polynucleotides encoding lgtE, the three exogenous polynucleotides encoding lgtA, and/or the three exogenous polynucleotides encoding lgtB are incorporated into the genome of the *E. coli*. In certain embodiments, one of the two exogenous polynucleotides encoding lst is expressed from a vector. In certain embodiments, the expression of lst is constitutively under control of lambda phage promoter PL in the vector. In certain embodiments, the exogenous polynucleotide encoding KdsB is expressed from a vector.

[00354] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce at least one glycan antigen (*e.g.*, genetic modifications of Section 5.2.1). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises genetic disruption of *waa*Q, *waa*G, *waa*P, *waa*S, *waa*B, *waa*O, *waa*R, *waaY, waaZ,* and *waa*U genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes. In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, the exogenous polynucleotide encoding lgtE, the exogenous polynucleotide encoding lgtA, the

exogenous polynucleotide encoding lgtB, and the exogenous polynucleotide encoding lst are incorporated into the genome of the *E. coli*. In certain embodiments, the exogenous polynucleotide encoding kdsB is expressed from a vector. In certain embodiments, the exogenous polynucleotide encoding Lst is expressed from a vector.

[00355] In certain embodiments, the *E. coli* comprises one or more genetic modifications to produce at least one glycan antigen (*e.g.*, genetic modifications of Section 5.2.1). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, at least one exogenous polynucleotide encoding lgtE, at least one exogenous polynucleotide encoding lgtA, at least one exogenous polynucleotide encoding lgtB, at least one exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, two exogenous polynucleotides each encoding lgtE, three exogenous polynucleotides each encoding lgtA, three exogenous polynucleotides each encoding lgtB, two exogenous polynucleotides each encoding lst, and an exogenous polynucleotide encoding kdsB. In certain embodiments, the *E. coli* comprises genetic disruption of *waa*Q, *waa*G, *waa*P, *waa*S, *waa*B, *waa*O, *waa*R, *waaY, waaZ*, and *waa*U genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes. In certain embodiments, the *E. coli* comprises genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes. In certain embodiments, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, one of the two exogenous polynucleotides encoding lst, the two exogenous polynucleotides encoding lgtE, the three exogenous polynucleotides encoding lgtA, and the three exogenous polynucleotides encoding lgtB are incorporated into the genome of the *E. coli*. In certain embodiments, one of the two exogenous polynucleotides encoding lst is expressed from a vector. In certain embodiments, the expression of lst is constitutively under control of lambda phage promoter PL in the vector. In certain embodiments, the exogenous polynucleotide encoding KdsB is expressed from a vector.

[00356] In certain embodiments, the *E. coli* comprises one or more genetic modifications to express at least one protein antigen (*e.g.*, genetic modifications of Section 5.2.2). In certain

embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding the MsrA/B polypeptide; an exogenous polynucleotide encoding the MetQ polypeptide; an exogenous polynucleotide encoding the AniA polypeptide; and/or an exogenous polynucleotide encoding the NHBA polypeptide. In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide; an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide; an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide; and/or an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide. In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6; an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and/or an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, the exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, the exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, and/or the exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide are expressed from a vector.

[00357] In certain embodiments, the *E. coli* comprises one or more genetic modifications to express at least one protein antigen (*e.g.*, genetic modifications of Section 5.2.2). In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding the MsrA/B polypeptide; an exogenous polynucleotide encoding the MetQ polypeptide; an exogenous polynucleotide encoding the AniA polypeptide; and an exogenous polynucleotide encoding the NHBA polypeptide. In certain embodiments, the *E. coli* comprises: an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide; an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide; an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide; and an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide. In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding the MsrA/B-

AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6; an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6. In certain embodiments, the exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, the exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, the exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, and the exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide are expressed from a vector.

[00358] In certain embodiments, the *E. coli* comprises one or more genetic modifications to genetic modifications for producing modified lipid A (*e.g.*, genetic modifications of Section 5.2.3). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and/or an exogenous polynucleotide encoding lpxE. In certain embodiments, the *E. coli* comprises genetic disruption *of msbB, eptA,* and/or *lpxT* genes. In certain embodiments of the *E. coli*, the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter. In certain embodiments, the exogenous polynucleotide encoding msbB, the exogenous polynucleotide encoding pagL, and/or the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*.

[00359] In certain embodiments, the *E. coli* comprises one or more genetic modifications to genetic modifications for producing modified lipid A (*e.g.*, genetic modifications of Section 5.2.3). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding pagL and/or an exogenous polynucleotide encoding lpxE. In certain embodiments, the *E. coli* comprises genetic disruption *of eptA* and/or *lpxT* genes. In certain embodiments of the *E. coli*, the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter. In certain embodiments, the exogenous polynucleotide encoding pagL and/or the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*.

[00360] In certain embodiments, the *E. coli* comprises one or more genetic modifications to genetic modifications for producing modified lipid A (*e.g.*, genetic modifications of Section 5.2.3). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding msbB, an

exogenous polynucleotide encoding pagL, and an exogenous polynucleotide encoding lpxE. In certain embodiments, the *E. coli* comprises genetic disruption of *msbB, eptA,* and *lpxT* genes. In certain embodiments of the *E. coli*, the promoter of pagP gene of the *E. coli* being replaced by a lambda PL promoter. In certain embodiments, the exogenous polynucleotide encoding msbB, the exogenous polynucleotide encoding pagL, and the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*.

[00361] In certain embodiments, the *E. coli* comprises one or more genetic modifications to genetic modifications for producing modified lipid A (*e.g.*, genetic modifications of Section 5.2.3). In certain embodiments, the *E. coli* comprises an exogenous polynucleotide encoding pagL and an exogenous polynucleotide encoding lpxE. In certain embodiments, the *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes. In certain embodiments of the *E. coli*, the promoter of pagP gene of the *E. coli* being replaced by a lambda PL promoter. In certain embodiments, the exogenous polynucleotide encoding pagL and the exogenous polynucleotide encoding lpxE are incorporated into the genome of the *E. coli*.

[00362] In certain embodiments, the *E. coli* comprises one or more genetic modifications to increase vesiculation. In certain embodiments, the *E. coli* further comprises genetic disruption of *ompA* gene, genetic disruption of *lpp* gene; and/or an exogenous polynucleotide encoding lpp. In certain embodiments, the *E. coli* further comprises genetic disruption of *ompA* gene, genetic disruption of *lpp* gene; and an exogenous polynucleotide encoding lpp. In certain embodiments, the exogenous polynucleotide encoding lpp is incorporated into the genome of the *E. coli*. In certain embodiments, the expression of lpp is driven by a pBAD promoter.

[00363] In certain embodiments, the *E. coli* further comprises genetic disruption of *fliC* gene.

[00364] In certain embodiments, the *E. coli* further comprises genetic disruption of *fim* gene cluster. In certain embodiments, the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00365] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding msbB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 22, an exogenous polynucleotide encoding pagL having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 23, and/or an exogenous polynucleotide encoding lpxE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *msbB, eptA,* and/or *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 33, an exogenous polynucleotide encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 35, an exogenous polynucleotide encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 37, and/or an exogenous polynucleotide encoding kdsB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and/or *waaU* genes ;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes ;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and/or

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00366] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding msbB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 22, an exogenous polynucleotide encoding pagL having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 23, and an exogenous polynucleotide encoding lpxE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *msbB*, *eptA*, and *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 33, an exogenous polynucleotide encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 35, an exogenous polynucleotide encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00367] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having at least about at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding pagL having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 23 and an exogenous polynucleotide encoding lpxE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *eptA* and *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 33, two exogenous polynucleotides each encoding lgtA having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 35, two exogenous polynucleotides each encoding lgtB having at least about 90% sequence identity to the amino acid sequence of SEQ

ID NO: 36, two exogenous polynucleotides each encoding lst having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00368] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding msbB having the amino acid sequence of SEQ ID NO: 22, an exogenous polynucleotide encoding pagL having the amino acid sequence of SEQ ID NO: 23, and/or an exogenous polynucleotide encoding lpxE having the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *msbB, eptA*, and/or *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;
(8) an exogenous polynucleotide encoding lpt3 having the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 33, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 35, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having the amino acid sequence of SEQ ID NO: 37, and/or an exogenous polynucleotide encoding kdsB having the amino acid sequence of SEQ ID NO: 53;
(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and/or *waaU* genes;
(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes;
(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes;
(12) genetic disruption of *ompA* gene;
(13) genetic disruption of *lpp* gene;
(14) an exogenous polynucleotide encoding lpp having the amino acid sequence of SEQ ID NO: 43;
(15) genetic disruption of *fliC* gene; and/or
(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00369] In certain embodiments, the *E. coli* provided herein comprises:
(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;
(2) an exogenous polynucleotide encoding a MetQ polypeptide having the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;
(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;
(5) an exogenous polynucleotide encoding msbB having the amino acid sequence of SEQ ID NO: 22, an exogenous polynucleotide encoding pagL having the amino acid sequence of SEQ ID NO: 23, and an exogenous polynucleotide encoding lpxE having the amino acid sequence of SEQ ID NO: 24;
(6) genetic disruption of *msbB, eptA*, and *lpxT* genes;
(7) the promoter of pagP gene of the *E. coli* being replaced by a lambda PL promoter;
(8) an exogenous polynucleotide encoding lpt3 having the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 33, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 35, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having the amino acid sequence of SEQ ID NO: 53;
(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;
(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;
(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes ;
(12) genetic disruption of *ompA* gene;
(13) genetic disruption of *lpp* gene;
(14) an exogenous polynucleotide encoding lpp having the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00370] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding msbB having the amino acid sequence of SEQ ID NO: 22, an exogenous polynucleotide encoding pagL having the amino acid sequence of SEQ ID NO: 23, and an exogenous polynucleotide encoding lpxE having the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *msbB, eptA*, and *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 33, two exogenous polynucleotides each encoding one copy of lgtA having the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 35, two exogenous polynucleotides each encoding one copy of lgtB having the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes ;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00371] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding pagL having the amino acid sequence of SEQ ID NO: 23, and an exogenous polynucleotide encoding lpxE having the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *eptA* and *lpxT* genes;

(7) the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having the amino acid sequence of SEQ ID NO: 30, an

exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 33, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 35, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 36, an exogenous polynucleotide encoding lst having the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes ;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00372] In certain embodiments, the *E. coli* provided herein comprises:

(1) an exogenous polynucleotide encoding a MsrA/B-AniA fusion polypeptide having the amino acid sequence of SEQ ID NO: 14 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(2) an exogenous polynucleotide encoding a MetQ polypeptide having the amino acid sequence of SEQ ID NO: 7 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(3) an exogenous polynucleotide encoding an AniA polypeptide having the amino acid sequence of SEQ ID NO: 4 and an AniA signal polypeptide having the amino acid sequence of SEQ ID NO: 3;

(4) an exogenous polynucleotide encoding a NHBA-MetQ fusion polypeptide having the amino acid sequence of SEQ ID NO: 21 and a MetQ signal polypeptide having the amino acid sequence of SEQ ID NO: 6;

(5) an exogenous polynucleotide encoding pagL having the amino acid sequence of SEQ ID NO: 23 and an exogenous polynucleotide encoding lpxE having the amino acid sequence of SEQ ID NO: 24;

(6) genetic disruption of *eptA* and *lpxT* genes;

(7) the promoter of pagP gene of the *E. coli* being replaced by a lambda PL promoter;

(8) an exogenous polynucleotide encoding lpt3 having the amino acid sequence of SEQ ID NO: 27, an exogenous polynucleotide encoding rfaK having the amino acid sequence of SEQ ID NO: 28, an exogenous polynucleotide encoding lgtF having the amino acid sequence of SEQ ID NO: 29, an exogenous polynucleotide encoding lgtG having the amino acid sequence of SEQ ID NO: 30, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 31, an exogenous polynucleotide encoding lgtE having the amino acid sequence of SEQ ID NO: 32, an exogenous polynucleotide encoding lgtA having the amino acid sequence of SEQ ID NO: 33, two exogenous polynucleotides each encoding lgtA having the amino acid sequence of SEQ ID NO: 34, an exogenous polynucleotide encoding lgtB having the amino acid sequence of SEQ ID NO: 35, two exogenous polynucleotides each encoding lgtB having the amino acid sequence of SEQ ID NO: 36, two exogenous polynucleotide each encoding lst having the amino acid sequence of SEQ ID NO: 37, and an exogenous polynucleotide encoding kdsB having the amino acid sequence of SEQ ID NO: 53;

(9) genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ*, and *waaU* genes;

(10) genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL* genes;

(11) genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes ;

(12) genetic disruption of *ompA* gene;

(13) genetic disruption of *lpp* gene;

(14) an exogenous polynucleotide encoding lpp having the amino acid sequence of SEQ ID NO: 43;

(15) genetic disruption of *fliC* gene; and

(16) genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

[00373] An exemplary scheme of the *E. coli* engineering is provided herein in Table 1 below:

Table 1: Genetic Modifications of Engineered *E. coli*

Genetic Modifications	Description and Functions
Engineered *E. coli* strain: StLTPro353 Original starting strain: *E. coli* C600	
Δ*msbB*::*msbB*	lipid A engineering
Δ*ompA*	vesiculation
Δ*lpp*	vesiculation
attλ:P_{BAD}_*lpp*	vesiculation
Δ*waaQ-U*: :*lgtA_lgtB_lgtE_lpt3_lgtF_rfaK_lgtG*	glycan antigen engineering *E. coli* LOS gene cluster has been replaced with one copy of the indicated genes.
Δ*wca-rfb*::P_{O16}-*lst_lgtB_lgtA_lgtE*	glycan antigen engineering *E. coli* adjacent gene cluster for colanic acid and O antigen biosynthesis have been replaced with the indicated genes. The constitutive promoter naturally driving the O antigen expression has been re-inserted to drive the expression of these genes. Multiple dosage has been identified as necessary for the optimal glycan conversion. The replaced genes clusters have been selected as they might create interference in the glycan biosynthesis and accumulate unwanted glycans on the OMV
Δ*wec*::*lgtB_lgtA*	glycan antigen engineering *E. coli* gene cluster for ECA (enterobacterial common antigen) has been replaced with a further copy of the indicated genes. The natural ECA constitutive promoter drives the expression of the genes. Multiple dosage has been identified as necessary for the optimal glycan conversion. The replaced genes clusters have been selected as they might create interference in the glycan biosynthesis and accumulate unwanted glycans on the OMV.
Δ*fliC*	further modifications
Δ *fim* gene cluster (*fimHGFDCIA*)	further modifications
Δ*eptA*::*pagL*	lipid A engineering
Δ*lpxT*::*lpxE*	lipid A engineering
ΔP_{pagP}::PL	lipid A engineering
Plasmid pLTPro159	Vector encoding Lst (pRESTmp_PL_lst) Backbone origin: pBAD backbone (pBBR ori, medium copy number). Trimethoprim resistance. Lambda phage promoter PL drives the expression of Lst. Optimal KDO conversion requires plasmid-borne lst expression
Plasmid pLTPro231 or pLTPro302	Vector encoding the encoding the four protein antigens and the KdsB. Backbone origin: pEXT20 (Dykxhoorn, et al. 1996) (ColE1 ori, high copy number). Resistance cassette exchanged from Ampicillin to Kanamycin. Plasmid-borne expression of KdsB is required for optimal KDO conversion. pLTPro231: Ptac (IPTG) promoter drives the expression of MsrA/B-AniA fusion polypeptide, MetQ polypeptide, AniA polypeptide, NHBA-MetQ fusion polypeptide, and KdsB.

Genetic Modifications	Description and Functions
	pLTPro302: Ptac (IPTG) promoter drives the expression of MsrA/B-AniA fusion polypeptide and NHBA-MetQ fusion polypeptide. J23105 promoter drives the expression of MetQ polypeptide, AniA polypeptide, and KdsB.

[00374] Plasmid maps of the expression vectors (as in Table 1) encoding the MsrA/B-AniA fusion polypeptide, MetQ polypeptide, AniA polypeptide, NHBA-MetQ fusion polypeptide, and KdsB are provided in **FIG. 1A** (pLTPro231) and **FIG. 1B** (pLTPro302). In certain embodiments, the *E. coli* comprises pLTPro231 disclosed herein. In certain embodiments, the *E. coli* comprises pLTPro302 disclosed herein.

[00375] Sequence information of the expression vectors (as in Table 1) encoding the MsrA/B-AniA fusion polypeptide, MetQ polypeptide, AniA polypeptide, NHBA-MetQ fusion polypeptide, and KdsB is provided in **FIG. 2A** (pLTPro231, SEQ ID NO: 66) and **FIG. 2B** (pLTPro302, SEQ ID NO: 67).

5.3 Pharmaceutical Compositions

[00376] In one aspect, the disclosure provides a therapeutic or pharmaceutical composition comprising engineered *E. coli* strains as described herein. In one aspect, the disclosure provides a therapeutic or pharmaceutical composition comprising an OMV vaccine as described herein. Vectors are described in detail above and would be known to one of skill in the art.

[00377] In certain embodiments, an OMV vaccine as described herein may be provided as a pharmaceutical or therapeutic composition to be administered to a subject or patient for treatment of *N. gonorrhoeae* infection.

[00378] In certain embodiments, a glycan antigen as described herein may be provided or administered to a subject or patient as OMV expressing the glycan antigen. In certain embodiments, a protein antigen as described herein may be provided or administered to a subject or patient as OMV expressing the protein antigen. The disclosure provides an OMV vaccine, pharmaceutical compositions and related methods of using these vaccines, compositions, or expression systems for inhibiting, preventing, or treating gonococcal and/or meningococcal infections. Also provided is a use of the polynucleotides, polypeptides, and expression vectors or systems described herein for the manufacture of a medicament to prevent or treat *N. gonorrhoeae* infections. The pharmaceutical composition can be either a therapeutic formulation or a prophylactic formulation. Typically, a pharmaceutical composition may contain one or more active ingredients and, optionally, some inactive ingredients. In certain embodiments, the active ingredient may be an OMV vaccine, glycan, recombinant polypeptide, an expression vector, or an expression system as described herein. In

some other embodiments, the active ingredient may include other antibacterial agents in addition to the expression system of the disclosure. The composition may additionally include one or more pharmaceutically acceptable vehicles and, optionally, other therapeutic ingredients (for example, antibiotics). Various pharmaceutically acceptable additives may also be used in such compositions.

[00379] In certain embodiments, an OMV vaccine for treatment of *N. gonorrhoeae* infections as described herein, and pharmaceutical compositions thereof, as described herein, may be administered in any appropriate dosage to obtain a therapeutic result. As would be understood by one of skill in the art, a dosage of OMV appropriate for treatment or prevention of *N. gonorrhoeae* infections or to achieve a particular outcome will vary depending on various factors including, but not limited to, the gene and promoter chosen, the condition, patient-specific parameters, *e.g.*, height, weight, and age, and whether prevention or treatment is to be achieved. An OMV vaccine of the disclosure may conveniently be provided in the form of formulations suitable for administration, *e.g.*, into the blood stream (*e.g.*, in an intracoronary artery). A suitable administration format may best be determined by a medical practitioner or clinician for each patient individually, according to standard procedures and may include, but is not limited to, intramuscular, buccal, rectal, intracoronary, intravenous, intranasal, trans-vaginal, subcutaneous, intra-arterial, intra-articular, intraperitoneal, parenteral or any other suitable mode of administration known in the art.

[00380] A vaccine or pharmaceutical composition of the disclosure may be prepared in accordance with standard procedures well known in the art. See, *e.g.*, Remington's Pharmaceutical Sciences, 19th Ed., Mack Publishing Company, Easton, Pa., 1995; Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978; U.S. Pat. Nos. 4,652,441; 4,917,893; 4,677,191; 4,728,721; and 4,675,189. Pharmaceutical compositions of the disclosure may be readily employed in a variety of therapeutic or prophylactic applications for preventing or treating gonococcal and/or meningococcal infections. For subjects at risk of developing a *N. gonorrhoeae* infection, a vaccine composition of the disclosure may be administered to provide prophylactic protection against *N. gonorrhoeae* infections. Depending on the specific subject and conditions, a composition of the disclosure may be administered to a subject or patient by a variety of administration modes known to the person of ordinary skill in the art, for example, intramuscular, subcutaneous, intravenous, intra-arterial, intra-articular, intraperitoneal, or parenteral routes. In certain embodiments, a composition as described herein may be administered to a subject in need of such treatment for a time and under conditions sufficient to prevent, inhibit, and/or ameliorate a selected disease or condition or one or more symptom(s) thereof. For therapeutic applications, a composition may contain a therapeutically effective amount of the

expression system described herein. For prophylactic applications, a composition as described herein may contain a prophylactically effective amount of an expression system as described herein. The appropriate amount of the expression system (*e.g.*, expression vectors) may be determined based on the specific disease or condition to be treated or prevented, severity, age of the subject, and other personal attributes of the specific subject (*e.g.*, the general state of the subject's health and the robustness of the subject's immune system). Determination of effective dosages may additionally be guided with animal model studies (*i.e.*, primate, canine, or the like), followed by human clinical trials, and by administration protocols that significantly reduce the occurrence or severity of targeted disease symptoms or conditions in the subject.

[00381] For prophylactic applications, an OMV vaccine as described herein may be provided in advance of any symptom, for example in advance of infection. A prophylactic administration of the immunogenic compositions may serve to prevent or ameliorate any subsequent infection. Thus, in certain embodiments, a subject to be treated is one who has, or is at risk for developing, a *N. gonorrhoeae* infections, for example because of exposure or the possibility of exposure to the bacterium. Following administration of a therapeutically effective amount of the disclosed therapeutic compositions, a subject or patient may be monitored for *N. gonorrhoeae* infections, symptoms associated with *N. gonorrhoeae* infections, or both.

[00382] For therapeutic applications, a composition as described herein may be provided at or after the onset of a symptom of disease or infection, for example after development of a symptom of *N. gonorrhoeae* infections, or after diagnosis of infection. A composition as described herein may thus be provided prior to the anticipated exposure to a N. gonococcal bacterial strain, so as to attenuate the anticipated severity, duration or extent of an infection and/or associated disease symptoms, after exposure or suspected exposure to the bacterium, or after the actual initiation of an infection.

[00383] In certain embodiments, an OMV vaccine of the disclosure may be provided in a dosage form containing an amount of OMV expressing or comprising a glycan antigen and one or more protein antigens that is effective in one or multiple doses. An effective dose may be any range deemed appropriate by a clinician or practitioner. Administration of an OMV vaccine with the glycan antigen and one or more protein antigens of the disclosure, a recombinant bacterial strain producing or expressing the glycan antigen and one or more protein antigens of the disclosure, or a composition comprising any of these may be in a buffer, such as phosphate-buffered saline, or other appropriate buffer or diluent. The amount of buffer or diluent may vary and would be determined by a clinician or practitioner.

[00384] In certain embodiments, a composition of the disclosure may be combined with other agents known in the art for treating or preventing *N. gonorrhoeae* infections. These may include any drug known or available in the art for treating a bacterial infection, *e.g.*, antibodies or other antibacterial agents such as antibacterial compounds or drugs, protease inhibitors, fusion protein inhibitors, or the like. In certain embodiments, a composition as described herein for treatment or prevention of *N. gonorrhoeae* infections may be advantageous in situations where a patient or subject is unresponsive to antibiotic treatment due to an increase in antibiotic resistance in the bacteria. Administration of a composition and one or more known anti-bacterial agent may be either concurrently or sequentially.

[00385] As described herein, OMV-based vaccines elicit higher titres of antibodies with broader reactivity than the corresponding recombinant proteins and may be more tolerable since less protein may be required to provide an effective protective antibody response. Thus, in certain embodiments, OMV may be administered with an adjuvant in order to enhance antibody responses. Suitable adjuvants are known in the art and can include, but are not limited to, aluminum compounds (*e.g.*, amorphous aluminum hydroxyphosphate sulfate (AAHS), aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate (Alum), aluminum hydroxide adjuvant (2% ALHYDROGEL)), cytosine phosphoguanine (CpG) nucleotides (*e.g.*, CpG 1018), AS01, AS04, QS-21, RIBI, MF59, or the like.

5.4 Methods of Making

[00386] In one aspect, the present disclosure provides a method for producing engineered *E. coli* strains as described herein. A number of methods have been reported to create gene knock-outs and gene knock-ins in *E. coli*. The most popular ones make use of the λ phage recombination system ("recombineering") that enormously enhances the double cross-over events between the chromosomal DNA and the transforming "donor DNA" designed to create the mutation (Murphy K C (1998) J. Bacteriol. 180, 2063). The donor DNA can be either synthetic single/double strand DNA or PCR-derived DNA (Ju et al., (2000) Proc. Natl. Acad. Sci. USA, 97, 5978; Ellis et al., (2001) Proc. Natl. Acad. Sci. USA, 98, 6742). The technology used for homologous recombination is also described in Datsenko et al. 2000. More recently, a combination of "recombineering" with CRISPR/Cas genome editing strategy has been shown to generate mutants in *E. coli* with high efficiency (Jiang et al. (2013) Nat. Biotechnol. 31, 233).

[00387] In certain embodiments, the bacterium is cultured in conditions suitable for growth and vesiculation, which include the use of rich media such as LB supplemented with additional carbon and nitrogen sources, or chemically defined media using different carbohydrates as carbon sources.

Growth temperatures typically vary from 20° C to 37° C and the supernatants containing the vesicles can be collected toward the end of the exponential phase or in the stationary phase of growth, depending upon the growth conditions in use. The conditions suitable for bacterial growth and vesiculation are known to anyone skilled in the art and are described for instance in Berlanda Scorza, F. et al. "High yield production process for Shigella outer membrane particles", PLoS One 7, e35616 (2012).

[00388] In one aspect, the present disclosure provides a method for producing an OMV as described herein. In certain embodiments, the OMV is secreted by an engineered *E. coli* as described in Section 5.2 above. In certain embodiments, the OMV is produced by an engineered *E. coli* as described in Section 5.2 above.

[00389] In certain embodiments, the method comprises the steps of: a) cultivating a bacteria host cell (*e.g.*, *E. coli*) comprising a nucleic acid molecule or an expression construct as described herein for expression in the host cell, b) extracting the OMV, and c) recovering the OMV, wherein the recovery at least comprises removal of the bacteria from the OMV. In certain embodiments, the recovery of the OMV in step c) is preceded by a step b), wherein the OMV are extracted.

[00390] In certain embodiments, the method comprises the first step of pre-cultures of the bacteria host cell (*e.g.*, *E. coli*) are inoculated from glycerol stock and grown overnight. In certain embodiments, bioreactor is inoculated with precultures. In certain embodiments, inducers are either present in the batch medium or absent. In certain embodiments, batch culture in bioreactor is interrupted 6 h after inoculum. In certain embodiments, the method comprises the next step of separating supernatant is from cells by centrifugation. In certain embodiments, supernatant is filtered through 0.45 um and 0.2 um filter, to completely separate from cell material. In certain embodiments, the supernatant can be stored at 4°C. In certain embodiments, the method comprises a further step of filtering the samples to filter away small impurities. In certain embodiments, pH is adjusted, the material undergoes TFF (tangential flow filtration) with 300 kDa cutoff, and buffer is exchanged. In certain embodiments, the sample is again filtered through 0.45 and 0.2 um filters. In certain embodiments, the sample undergoes a last purification step through a SEC chromatography. In certain embodiments, the sample undergoes TFF and diafiltration for final concentration and buffer adjustment.

[00391] In certain embodiments, the expression of protein antigens on the OMVs are assessed, *e.g.*, by immunoblots or ELISAs. In certain embodiments, the expression of glycan antigens on the OMVs are assessed, *e.g.*, by immunoblots or ELISAs.

[00392] In certain embodiments, the four protein antigens as described herein together amount to approximately 3 to 10% of the total OMV protein.

[00393] In certain embodiments, the composition produced by the method disclosed herein has a purity of the OMV of at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 99%, where the purity is calculated as the percentage of the area under the OMV containing peak compared to the area under the entire chromatogram as measured by SEC-HPLC. In certain embodiments, the composition produced by the method disclosed herein has a purity of the OMV of at least 99%, where the purity is calculated as the percentage of the area under the OMV containing peak compared to the area under the entire chromatogram as measured by SEC-HPLC. In certain embodiments, the composition produced by the method disclosed herein does not comprise an aggregation of the OMV as assessed by SEC-HPLC.

[00394] In certain embodiments, the composition produced by the method disclosed herein is essentially free of DNA from *E coli*. In certain embodiments, the composition comprises less than 5 ng, less than 4 ng, less than 3 ng, less than 1 ng, less than 100 pg, less than 50 pg, less than 25 pg, less than 10 pg, less than 5 pg, or less than 1.5 pg, DNA from *E coli* /μg total protein as measure by qPCR. In certain embodiments, the composition comprises less than 1.5 pg, DNA from *E coli* /μg total protein as measure by qPCR.

[00395] In certain embodiments, the diameter of the OMV in the composition produced by the method disclosed herein, as measured by DLS, is about 30 nm, about 35 nm, about 40 nm, about 45 mg (*e.g.*, 44.5 nm), about 50 nm, about 55 nm, or about 60 nm. In certain embodiments, the diameter of the OMVs in the composition produced by the method disclosed herein is between 20 nm and 80 nm, between 30 nm and 70 nm, between 40 nm and 60 nm, or between 50 nm and 60 nm as measured by DLS.

[00396] In certain embodiments, no significant degradation of OMV in the composition produced by the method disclosed herein is observed after being stored at 2°C - 8°C for at least about 4 week, at least about 8 week, at least about 12 week, at least about 16 week, at least about 20 week, at least about 24 week, at least about 28 week, at least about 32 week, at least about 36 week, at least about 40 week, at least about 44 week, at least about 48 week, at least about 52 week, at least about 56 week, at least about 1 year, or more.

5.5 Methods of Using

[00397] The present disclosure also provides a method for inducing an immune response against *N. gonococcal* in a subject in need thereof, comprising administering an effective amount of an OMV as described herein (see Section 5.1) to the subject. In certain embodiments,

administration of the OMV stimulates immune response against the protein antigen or glycan antigen disclosed herein. In certain embodiments, administration of the OMV stimulates immune response against the *N. gonococcal*. In certain embodiments, administration of the OMV stimulates a strong immune response against the *N. gonorrhoeae*. In certain embodiments, administration of the OMV stimulates a safe immune response. For example, in certain embodiments, administration of an OMV vaccine as described herein do not cause temperature increase in a subject receiving the OMVs. Exemplary assays to measure the immune responses elicited by OMV vaccine are provided in Section 7.

[00398] In certain embodiments, the stimulated immune response is against the bacteria (*e.g.*, *N. gonococcal* bacterial strain) from which the antigens of the OMVs are derived from. In certain embodiments, the stimulated immune response comprises inducing antibodies against the protein and/or glycan antigens disclosed herein. In certain embodiments, the induced antibodies are able to trigger the killing of the bacteria (*e.g.*, *N. gonococcal* bacterial strain). In certain embodiments, the induced antibodies are able to trigger the complement-mediated killing of the bacteria (*e.g.*, *N. gonococcal* bacterial strain). Exemplary assays for measuring the immunogenicity and for measuring the bacterial killing ability (*e.g.*, serum bactericidal assay (SBA)) are provided in Section 7.

[00399] In certain embodiments, the induced antibodies are able to interfere with adherence of bacteria (*e.g.*, *N. gonococcal* bacterial strain) to cells. In certain embodiments, the cells are epithelial cells. In certain embodiments, the cells are genital tract epithelial cells, for example, urethral epithelial cells or cervical epithelial cells. In certain embodiments, the cells are pharyngeal epithelial cells. Exemplary adherence assays for measuring the ability to interfere with the adherence of bacteria to cells are provided in Section 7.

[00400] In certain embodiments, the induced antibodies block MsrA/B by blocking the binding to its substrate methylsulfoxide (Met(O)) or oxidized peptide (Pep-O; VAPVGMHIANGM (SEQ ID NO: 69) from *N.gonorrhoeae* OMP). In certain embodiments, the induced antibodies block MetQ by blocking the binding to methionine (Met) and seleniomethionine (SeMet). In certain embodiments, the induced antibodies block AniA by blocking the binding to its substrate AccA (AniA Cu chaperone A) peptide 17 (GSYHVMFMGLKKQLK (SEQ ID NO: 70)). Exemplary assays (*e.g.*, Surface Plasmon Resonance (SPR)) for measuring the ability to interfere with the adherence of bacteria to cells are provided in Section 7.

[00401] In certain embodiments, the *N. gonococcal* bacterial strain is a WHO F strain, a WHO Z strain, a WHO R strain, a WHO K strain, a 1291 strain, a 1263546 strain, a 1087092 strain, a WHO S2 strain, or a FA1090 strain.

[00402] The present disclosure also provides a method for treating *N. gonorrhoeae* infection in a subject in need thereof, comprising administering an effective amount of an OMV as described herein (see Section 5.1) to the subject. In one aspect, the present disclosure provides a method for preventing *N. gonorrhoeae* infection in a subject in need thereof, comprising administering an effective amount of an OMV as described herein (see Section 5.1) to the subject. In certain embodiments, such administration of an OMV vaccine may be therapeutic and result in amelioration of symptoms associated with *N. gonorrhoeae* infection in a patient. In other embodiments, such administration of an OMV vaccine may be prophylactic and result in prevention of infection and development of disease.

[00403] A method of the present disclosure may treat or prevent infection of a subject or patient with *N. gonorrhoeae* infection as described herein. Administration of a composition comprising an OMV as described herein may be in a clinical setting as described herein, or may be in an alternate setting as deemed appropriate by a clinician or practitioner. Further embodiments for administration of such OMV vaccines are described herein elsewhere.

[00404] In certain embodiments, such a composition comprising an OMV vaccine as described herein may be combined with other therapies or treatments for treatment of *N. gonorrhoeae* infection in a patient. Any appropriate drug treatment or therapeutic modality may be used as deemed appropriate by a clinician.

[00405] Administration of an OMV vaccine as described herein may reduce the number of days of *N. gonorrhoeae* infection symptoms by one or more days, such as reducing symptoms by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or the like. Administration of an OMV vaccine as described herein may be in a single administration or dose, or may be in more than one administration or dose, such as including 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more doses. As would be understood by one of skill in the art, some patients or subjects may benefit from more than one administration or treatment with an OMV vaccine of the present disclosure. Such determination would be made by a clinician or other qualified healthcare personnel.

[00406] In other embodiments, symptoms of *N. gonorrhoeae* infection may be reduced by one week or more, such as including, but not limited to, one week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, or 8 weeks, or more. In other embodiments, administration of an OMV vaccine

as described herein may reduce the severity or duration of *N. gonorrhoeae* infection by 10%, or 20%, or 30%, or 40%, or 50%, or 60%, or 70%, or 80%, or 90%, or 100%.

[00407] Unless otherwise specified herein, the methods described herein can be performed in accordance with the procedures exemplified herein or routinely practiced methods well known in the art. See, *e.g.*, Methods in Enzymology, Volume 289: Solid-Phase Peptide Synthesis, J. N. Abelson, M. I. Simon, G. B. Fields (Editors), Academic Press; 1st edition (1997) (ISBN-13: 978-0121821906); U.S. Pat. Nos. 4,965,343, and 5,849,954; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y., (3rd ed., 2000); Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (2003); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol. 152, S. L. Berger and A. R. Kimmel Eds., Academic Press Inc., San Diego, USA (1987); Current Protocols in Protein Science (CPPS) (John *E. coli*gan, et. al., ed., John Wiley and Sons, Inc.), Current Protocols in Cell Biology (CPCB) (Juan S. Bonifacino et. al. ed., John Wiley and Sons, Inc.), and Culture of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, Publisher: Wiley-Liss; 5th edition (2005), Animal Cell Culture Methods (Methods in Cell Biology, Vol. 57, Jennie P. Mather and David Barnes editors, Academic Press, 1st edition, 1998).

5.6 Kits

[00408] The disclosure further provides a kit comprising one or more single-use containers comprising engineered *E. coli* strains as described herein. The disclosure further provides a kit comprising one or more single-use containers comprising an OMV vaccine as described herein. In certain embodiments, a kit of the disclosure may provide a composition comprising an OMV vaccine for treatment or prevention of *N. gonorrhoeae* infection as described herein. In other embodiments, a kit as described herein may provide a bacterial strain as described herein, for example, in culture or as a frozen stock combined with, *e.g.*, glycerol. In certain embodiments, a kit may provide a pharmaceutical composition comprising an OMV vaccine as described herein, for administration to a subject or patient. In other embodiments, sterile reagents and/or supplies for administration of an OMV vaccine, purified gonococcal protein, RNA, vectors, and/or pharmaceutical composition as described herein, may be provided as appropriate. A kit may further comprise reagents for cell transformation and/or transfection, bacterial or viral culture, or the like.

[00409] Components provided in a kit of the disclosure may include, for example, any starting materials useful for performing a method as described herein. Such a kit may comprise one or more such reagents or components for use in a variety of assays, including for example, nucleic acid

assays, *e.g.*, PCR or RT-PCR assays, luciferase (Luc) assays, cell transformation/ transfection, viral/cell culture, blood assays, *i.e.*, complete blood count (CBC), viral titer/viral load assays, antibody assays, viral antigen detection assays, DNA or RNA detection assays, bacterial titer assays, virus neutralization assays, genetic complementation assays, or any assay useful in accordance with the disclosure. Components may be provided in lyophilized, desiccated, or dried form as appropriate, or may be provided in an aqueous solution or other liquid media appropriate for use in accordance with the disclosure.

[00410] Kits useful for the present disclosure may also include additional reagents, *e.g.*, buffers, substrates, antibodies, ligands, detection reagents, media components, such as salts including MgCh, a polymerase enzyme, deoxyribonucleotides, ribonucleotides, expression vectors, and the like, reagents for DNA isolation, DNA/RNA transfection, or the like, as described herein. Such reagents or components are well known in the art. In certain embodiments, one or more adjuvants described herein may be included with a kit of the present disclosure. Where appropriate, reagents included with such a kit may be provided either in the same container or media as a primer pair or multiple primer pairs. In certain embodiments, such reagents may be placed in a second or additional distinct container into which an additional composition or reagents may be placed and suitably aliquoted. Alternatively, reagents may be provided in a single container means. A kit of the disclosure may also include packaging components, instructions for use, including storage requirements for individual components as appropriate. Such a kit as described herein may be formulated for use in a clinical setting, such as a hospital, treatment center, or clinical setting, or may be formulated for personal use as appropriate.

6. SEQUENCES

SEQ ID NO.	Description	Sequence
1	Full length AniA polypeptide amino acid sequence (with signal peptide) (GenBank WP_241499695.1)	MKRQALAAMIASLFALAACGGEQAAQAPAETPAASAEAASSAAQATAETPAGE LPVIDAVTTHAPEVPPAIDRDYPAKVRVKMETVEKTMKMDDGVEYRYWTFDGD VPGRMIRVREGDTVEVEFSNNPSSTVPHNVDFHAATGQGGGAAATFTAPGRTS TFSFKALQPGLYIYHCAVAPVGMHIANGMYGLILVEPKEGLPKVDKEFYIVQG DFYTKGKKGAQGLQPFDMDKAVAEQPEYVVFNGHVGAIAGDNALKAKAGETVR MYVGNGGPNLVSSFHVIGEIFDKVYVEGGKLINENVQSTIVPAGGSAIVEFKV DIPGNYTLVDHSIFRAFNKGALGQLKVEGAENPEIMTQKLSDTAYAGSGAASA PAASAPAASAPAASAPAASAPAASASEKSVY
2	Exemplary AniA polypeptide amino acid	MKRQALAAMIASLFALAACGGEQAAQAPAETPAASAEAASSAAQATAETPAGE LPVIDAVTTHAPEVPPAIDRDYPAKVRVKMETVEKTMKMDDGVEYRYWTFDGD VPGRMIRVREGDTVEVEFSNNPSSTVPHNVDFHAATGQGGGAAATFTAPGRTS TFSFKALQPGLYIYHCAVAPVGMHIANGMYGLILVEPKEGLPKVDKEFYIVQG

SEQ ID NO.	Description	Sequence
	sequence (with signal peptide)	DFYTKGKKGAQGLQPFDMDKAVAEQPEYVVFNGHVGAIAGDNALKAKAGETVR MYVGNGGPNLVSSFHVIGEIFDKVYVEGGKLINENVQSTIVPAGGSAIVEFKV DIPGNYTLVDHSIFRAFNKGALGQLKVE
3	AniA signal peptide amino acid sequence	MKRQALAAMIASLFALAA
4	Exemplary AniA polypeptide amino acid sequence (without signal peptide)	CGGEQAAQAPAETPAASAEAASSAAQATAETPAGELPVIDAVTTHAPEVPPAI DRDYPAKVRVKMETVEKTMKMDDGVEYRYWTFDGDVPGRMIRVREGDTVEVEF SNNPSSTVPHNVDFHAATGQGGGAAATFTAPGRTSTFSFKALQPGLYIYHCAV APVGMHIANGMYGLILVEPKEGLPKVDKEFYIVQGDFYTKGKKGAQGLQPFDM DKAVAEQPEYVVFNGHVGAIAGDNALKAKAGETVRMYVGNGGPNLVSSFHVIG EIFDKVYVEGGKLINENVQSTIVPAGGSAIVEFKVDIPGNYTLVDHSIFRAFN KGALGQLKVE
5	Full length MetQ polypeptide amino acid sequence (with signal peptide) (GenBank: WP_003687141.1)	MKTFFKTLSAAALALILAACGGQKDSAPAASAAAPSADNGAAKKEIVFGTTVG DFGDMVKEQIQAELEKKGYTVKLVEFTDYVRPNLALAEGELDINVFQHKPYLD DFKKEHNLDITEAFQVPTAPLGLYPGKLKSLEEVKDGSTVSAPNDPSNFARAL VMLNELGWIKLKDGINPLTASKADIAENLKNIKIVELEAAQLPRSRADVDFAV VNGNYAISSGMKLTEALFQEPSFAYVNWSAVKTADKDSQWLKDVTEAYNSDAF KAYAHKRFEGYKYPAAWNEGAAK
6	MetQ signal peptide amino acid sequence	MKTFFKTLSAAALALILAA
7	Exemplary MetQ polypeptide amino acid sequence (without signal peptide)	CGGQKDSAPAASAAAPSADNGAAKKEIVFGTTVGDFGDMVKEQIQAELEKKGY TVKLVEFTDYVRPNLALAEGELDINVFQHKPYLDDFKKEHNLDITEAFQVPTA PLGLYPGKLKSLEEVKDGSTVSAPNDPSNFARALVMLNELGWIKLKDGINPLT ASKADIAENLKNIKIVELEAAQLPRSRADVDFAVVNGNYAISSGMKLTEALFQ EPSFAYVNWSAVKTADKDSQWLKDVTEAYNSDAFKAYAHKRFEGYKYPAAWNE GAAK
8	Full length MsrA/B polypeptide amino acid sequence (GenBank MDO6098055.1)	MKHRTFFSLCAKFGCLLALGACSPKIVDAGTATVPHTLSTLKTADNRPASVYL KKDKPTLIKFWASWCPLCLSELGQAEKWAQDAKFSSANLITVASPGFLHEKKD GEFQKWYAGLNYPKLPVVTDNGGTIAQNLNISVYPSWALIGKDGDVQRIVKGS INEAQALALIRNPNADLGSLKHSFYKPDTQKKDSAIMNTRTIYLAGGCFWGLE AYFQRIDGVVDAVSGYANGNTENPSYEDVSYRHTGHAETVKVTYDADKLSLDD ILQYYFRVVDPTSLNKQGNDTGTQYRSGVYYTDPAEKAVIAAALKREQQKYQL PLVVENEPLKNFYDAEEYHQDYLIKNPNGYCHIDIRKADEPLPGKTKAAPQGK GFDAATYKKPSDAELKRTLTEEQYQVTQNSATEYAFSHEYDHLFKPGIYVDVV SGEPLFSSADKYDSGCGWPSFTRPIDAKSVTEHDDFSFNMRRTEVRSRAADSH LGHVFPDGPRDKGGLRYCINGASLKFIPLEQMDAAGYGALKGKVK
9	Exemplary MsrA/B polypeptide amino acid sequence of MsrA/B-AniA fusion polypeptide	PHTLSTLKTADNRPASVYLKKDKPTLIKFWASWCPLCLSELGQAEKWAQDAKF SSANLITVASPGFLHEKKDGEFQKWYAGLNYPKLPVVTDNGGTIAQNLNISVY PSWALIGKDGDVQRIVKGSINEAQALALIRNPNADLGSLKHS

SEQ ID NO.	Description	Sequence
10	Exemplary AniA polypeptide amino acid sequence (with signal peptide) of MsrA/B-AniA fusion polypeptide	MKRQALAAMIASLFALAACGGEQAAQAPAETPAASAEAASSAAQATAETPA
12	Exemplary AniA polypeptide amino acid sequence (without signal peptide) of MsrA/B-AniA fusion polypeptide	CGGEQAAQAPAETPAASAEAASSAAQATAETPA
13	Exemplary MsrA/B-AniA fusion polypeptide amino acid sequence (with signal peptide)	MKRQALAAMIASLFALAACGGEQAAQAPAETPAASAEAASSAAQATAETPAPH TLSTLKTADNRPASVYLKKDKPTLIKFWASWCPLCLSELGQAEKWAQDAKFSS ANLITVASPGFLHEKKDGEFQKWYAGLNYPKLPVVTDNGGTIAQNLNISVYPS WALIGKDGDVQRIVKGSINEAQALALIRNPNADLGSLKHS
14	Exemplary MsrA/B-AniA fusion polypeptide amino acid sequence (without signal peptide)	CGGEQAAQAPAETPAASAEAASSAAQATAETPAPHTLSTLKTADNRPASVYLK KDKPTLIKFWASWCPLCLSELGQAEKWAQDAKFSSANLITVASPGFLHEKKDG EFQKWYAGLNYPKLPVVTDNGGTIAQNLNISVYPSWALIGKDGDVQRIVKGSI NEAQALALIRNPNADLGSLKHS
15	Full length NHBA polypeptide amino acid sequence (GenBank QXN37363.1)	MFKRSVIAMACIFPLSACGGGGGGSPDVKSADTPSKPAAPVVAENAGEGVLPK EKKDEEAAGGAPQADTQDATAGEGSQDMAAVSAENTGNGGAATTDNPKNEDAG AQNDMPQNAAESANQTGNNQPAGSSDSAPASNPAPANGGSDFGRTNVGNSVVI DGPSQNITLTHCKGDPCNGDNLLDEEAPPKSEFESLSDEEKIKKYKKDGEKFT GLVAIKVENNGLNKYTIIYQAQPTRSARSRRSLPAEIPLIPVNQADTLIVDGE AVSLTGHSGNIFAPEGNYRYLTYGAEKLPGGSYALRVQGEPAKGEMLAGTAVY NGEVLHFHMENGRPYPSGGRFAAKVDFGSKSVDGIIDSSDDLHMGTQKFKAAI DGNGFKGTWTENGGGDVSGRFYGPAGEEVAGKYSYRPTDAEKGGFGVFAGKKD RD
16	Exemplary NHBA polypeptide amino acid sequence of NHBA-MetQ fusion polypeptide; Exemplary cNHBA polypeptide amino acid	SLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPGG SYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFGSKS VDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEEVAG KYSYRPTDAEKGGFGVFAGKKDRD

SEQ ID NO.	Description	Sequence
	sequence (without signal peptide)	
17	Exemplary MetQ polypeptide amino acid sequence (with signal peptide) of NHBA-MetQ fusion polypeptide	MKTFFKTLSAAALALILAACGGQKDSAPAASAAAPSADNGAA
19	Exemplary MetQ polypeptide amino acid sequence (without signal peptide) of NHBA-MetQ fusion polypeptide	CGGQKDSAPAASAAAPSADNGAA
20	Exemplary NHBA-MetQ fusion polypeptide (with signal peptide)	MKTFFKTLSAAALALILAACGGQKDSAPAASAAAPSADNGAASLPAEIPLIPV NQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPGGSYALRVQGEPA KGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFGSKSVDGIIDSSDDL HMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEEVAGKYSYRPTDAEK GGFGVFAGKKDRD
21	Exemplary NHBA-MetQ fusion polypeptide (without signal peptide)	CGGQKDSAPAASAAAPSADNGAASLPAEIPLIPVNQADTLIVDGEAVSLTGHS GNIFAPEGNYRYLTYGAEKLPGGSYALRVQGEPAKGEMLAGTAVYNGEVLHFH MENGRPYPSGGRFAAKVDFGSKSVDGIIDSSDDLHMGTQKFKAAIDGNGFKGT WTENGGGDVSGRFYGPAGEEVAGKYSYRPTDAEKGGFGVFAGKKDRD
22	msbB (from *E. coli* C600) amino acid sequence (GenBank WP_000448381)	METKKNNSEYIPEFDKSFRHPRYWGAWLGVAAMAGIALTPPKFRDPILARLGR FAGRLGKSSRRRALINLSLCFPERSEAEREAIVDEMFATAPQAMAMMAELAIR GPEKIQPRVDWQGLEIIEEMRRNNEKVIFLVPHGWAVDIPAMLMASQGQKMAA MFHNQGNPVFDYVWNTVRRRFGGRLHARNDGIKPFIQSVRQGYWGYYLPDQDH GPEHSEFVDFFATYKATLPAIGRLMKVCRARVVPLFPIYDGKTHRLTIQVRPP MDDLLEADDHTIARRMNEEVEIFVGPRPEQYTWILKLLKTRKPGEIQPYKRKD LYPIK
23	pagL from Salmonella enterica ssp. enterica serovar Typhimurium 14028S (GenBank WP_000281877)	MYMKRIFIYLLLPCAFACSANDNVFFGKGNKHQISFAAGESIRRGGVEHLYTA FLTYSEPSDFFFLQARNNLELGGFKAKGSDDCSKHSGSVPCNKYNQGVLGISK DVALVHFAGIYTGIGLGAYIKSKSRDDMRVNSAFTFGEKAFLGWNFGAFSTEA YIRHFSNGSLTDKNSGHNFVGASISYNF
24	lpxE from Francisella tularensis ssp. Novicida U112	MLKQTLQTNFQGFKDIFKKPKLHNHKLPRYLQLKYTFIPLLILVIFAYYNLDT PVENYIKHSMPNIVGVIFGKITDVGKAEYILIICGVIVLARLFTDSQKLSANT RAMFDKVSAYAGFILATVAISGILGQILKMIIGRARPKFFLEYGSHYFQHFHA

SEQ ID NO.	Description	Sequence
	(GenBank AAU11503)	PGYDFASMPSGHSITVGAMFIAFFYIFPKLRYFWYLLIVVFAGSRIMVGSHYP SDVIFGVAFGCYCTAYIYYWMRNREII
25	PagP promoter sequence before substitution with lambda PL promoter (start of PagP ORF underlined)	cgcatcatctttaatcgatgcgCGGAAATATTTAACTTGAACAAGCGGAAATA AATAGAGCAGCTATTCAGATTATTCTTTATGTTGGGTCTATTAAGGTTATGTT AATTGTAGCTTTGCTATGCTAGTAGTAGATTTTTGATAAATGTTTTATGGTCA CAAatgaacgtg
26	After substitution with lambda PL promoter (PL promoter bolded, FRT site double underlined, start of PagP ORF underlined)	cgcatcatctttaatcgatgcgATCGCCCGCGAAGTTCCTATTCCGAAGTTCC TATTCTATCAGAAGTATAGGAACTTCCGGCCGGCCTTCTTTGA**TTATCTCTGG CGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCA**CATCAGCAGGACGC ACTGACCGTAATTATATTTATAAGGAGGTAATTCTCGAGatgaacgtg
27	Lpt3 from Neisseria meningitidis C311 (GenBank QXZ29326)	MMNKSFLTLVLYSSLLTASEIAYRFVFGIETLPAAKIAETFALTFVIAALYLF ARYKVTRLLIAVFFAFSIIANNVHYAVYQSWMTGINYWLMLKEVTEVGSAGAS MLDKLWLPVLWGVLEVMLFCSLAKFRRKTHFSADILFAFLMLMIFVRSFDTKQ EHGISPKPTYSRIKANYFSFGYFVGRVLPYQLFDLSRIPAFKQPAPSKIGQGS VQNIVLIMGESESAAHLKLFGYGRETSPFLTRLSQADFKPIVKQSYSAGFMTA VSLPSFFNAIPHANGLEQISGGDTNMFRLAKEQGYETYFYSAQAENEMAILNL IGKKWIDHLIQPTQLGYGNGDNMPDEKLLPLFDKINLQQGKHFIVLHQRGSHA PYGALLQPQDKVFGEADIVDKYDNTIHKTDQMIQTVFEQLQKQPDGNWLFAYT SDHGQYVRQDIYNQGTVQPDSYLVPLVLYSPDKAVQQAANQAFAPCEIAFHQQ LSTFLIHTLGYDMPVSGCREGSVTGNLITGDAGSLNIRDGKAEYVYPQ
28	RfaK (waaQ-U locus) from Neisseria meningitidis MC58 (GenBank AAF42053)	MEKEFRILNIVSAKIWGGGEQYVYDVSKALGLRGCTMFTAVNKNNELMHRRFS EVSSVFTTRLHTLNGLFSLYALTRFIRKNRISHLMIHTGKIAALSILLKKLTG VRLIFVKHNVVANKTDFYHRLIQKNTDRFICVSRLVYDVQTADNPFKEKYRIV HNGIDTGRFPPSQEKPDSRFFTVAYAGRISPEKGLENLIEACVILHRKYPQIR LKLAGDGHPDYMCRLKRDVSASGAEPFVSFEGFTEKLASFYRQSDVVVLPSLV PEAFGLSLCEAMYCRTAVISNTLGAQKEIVEHHQSGILLDRLTPESLADEIER LVLNPETKNALATAAHQCVAARFTINHTADKLLDAI
29	LgtF (waaQ-U locus) from Neisseria meningitidis MC58 (GenBank AAF42052)	MKKVSVLIVAKNEANHIQECIESCRFDKEVIVIDDYSTDNTAEIAEGLGAKVF RRHLNGDFGAQKTFAIEQAGGEWVFLIDADERCTPELSDEISKIVQTGDYAAY FVERRNLFPNHPATHGAMRPDSVCRLMPKKDSSVQGKVHETVQTPYPKRRLKH FMYHYTYDNWEQYFNKFNKYTSISAEKYREQGKPVRFVRDIILRPIWGFFKIY ILNKGFLDGKMGWIMSVNHSYYTMIKYVKLYYLYKSGGKF
30	LgtG (waaQ-U locus) from Neisseria meningitidis M22790 (GenBank AOA42063)	MKLKIDIATNNFKHGGGTERYTLDLVKGLNRQNITPAVYATKFDHSIPEYALI EPHLVDQHRTLKKLRSFLFSSRLTQTRKNSAAKLIACHHADYADLLICGGTHL GYLHHMAQKPNLLDRLAIRRNRSNYATAKLIMAHSHMMRRELVGLYGVPPERI QVAPPPADTERFFPQPGETADLRAKYGFADYETVFLFPSTGHTRKGLELLADF FEHTGLPVKLAVVGSPLPRPMKNVVGLGFCTDMPELYRAADFTIMASLYEPFG

SEQ ID NO.	Description	Sequence
		LVGVESVLCGTRVVLSENMACTEVMNEEAGFFFSRQNPETLAQAVAQAVSLKK QGGHRLSDPMRALNYNPSLSHHIDRLTDMLASV
31	LgtE (waaQ-U locus) from Neisseria gonorrhoeae FA19 (GenBank AKP11657)	MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLEQAMAELVPGLS AHPYLSGVEKACFMSHAVLWEQALDEGLPYIAVFEDDVLLGEGAEQFLAEDTW LEERFDKDSAFIVRLETMFAKVIVRPDKVLNYENRSFPLLESEHCGTAGYIIS REAMRFFLDRFAVLPPERIKAVDLMMFTYFFDKEGMPVYQVSPALCTQELHYA KFLSQNSMLGSDLEKDREQGRRHRRSLKVMFDLKRALGKFGREKKKRMERQRQ AELEKVYGRRVILFK
32	LgtE (wca-rfb locus) from Neisseria gonorrhoeae 1291 (GenBank AAF14363)	MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLERAMAELVPGLS AHPYLSGVEKACFMSHAVLWKQALDEGVPYVAVFEDDVLFGKDAEKFLAEDTW LEERFDKDSAFVVRLETMFAKVIVRPDKVLNYENRSFPLLESEHCGTAGYIIS REAMRFFLDRFAVLPAERIKAVDLMMFTYFFDKEGMPVYQVSPALCTQELHYA KFLSQNSMLGSDLEKDREQGRRHRRSLKAMFDLKRALGKFGREKNKTMESQRQ AELEKVYGRRVILFK
33	LgtA (waaQ-U locus) from Neisseria meningitidis MC58 (GenBank AAF42258)	MPSEAFRRHRAYRENKLQPLVSVLICAYNVEKYFAQSLAAVVNQTWRNLDILI VDDGSTDGTLAIAQRFQEQDGRIRILAQPRNSGLIPSLNIGLDELAKSGGGGE YIARTDADDIAAPDWIEKIVGEMEKDRSIIAMGAWLEVLSEEKDGNRLARHHE HGKIWKKPTRHEDIADFFPFGNPIHNNTMIMRRSVIDGGLRYNTERDWAEDYQ FWYDVSKLGRLAYYPEALVKYRLHANQVSSKYSIRQHEIAQGIQKTARNDFLQ SMGFKTRFDSLEYRQIKAVAYELLEKHLPEEDFERARRFLYQCFKRTDTLPAG AWLDFAADGRMRRLFTLRQYFGILHRLLKNR
34	LgtA (wca-rfb locus and wec locus) from Neisseria gonorrhoeae 1291 (GenBank AAF14359)	MQPLVSVLICAYNVEKYFAQSLAAVVNQTWRNLDILIVDDGSTDGTLAIAKDF QKRDSRIKILAQAQNSGLIPSLNIGLDELAKSGGGGYIARTDADDIASPGWIE KIVGEMEKDRSIIAMGAWLEILSEEKDGNRLARHHKHGKIWKKPTRHEDIAAF FPFGNPIHNNTMIMRRSVIDGGLRYNTGRDWAEDYQFWYDVSKLGRLAYYPEA LVKYRLHANQVSSKHSVRQHEIAQGIQKTARNDFLQSMGFKTRFDSLEYRQTK AAAYELPEKDLPEEDFERARRFLYQCFKRTDTPPSGAWLDFAADGRMRRLFTL RQYFGILYRLIKNRRQARSDSAGKEQEI
35	LgtB (waaQ-U locus) from Neisseria meningitidis MC58 (GenBank AAF42257)	MQNHVISLASAAERRAHIADTFGRHGIPFQFFDALMPSERLEQAMAELVPGLS AHPYLSGVEKACFMSHAVLWKQALDEGLPYITVFEDDVLLGEGAEKFLAEDAW LQERFDPDTAFIVRLETMFMHVLTSPSGVADYCGRAFPLLESEHWGTAGYIIS RKAMRFFLDRFAALPPEGLHPVDLMMFSDFFDREGMPVCQLNPALCAQELHYA KFHDQNSALGSLIEHDRLLNRKQQRRDSPANTFKHRLIRALTKISREREKRRQ RREQFIVPFQ
36	LgtB (wca-rfb locus and wec locus) from Neisseria gonorrhoeae 1291 (GenBank AAF14360)	MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLERAMAELVPGLS AHPYLSGVEKACFMSHAVLWEQALDEGVPYIAVFEDDVLLGEGAEQFLAEDTW LQERFDPDSAFVVRLETMFMHVLTSPSGVADYGGRAFPLLESEHCGTAGYIIS RKAMRFFLDRFAVLPPERLHPVDLMMFGNPDDREGMPVCQLNPALCAQELHYA KFHDQNSALGSLIEHDRRLNRKQQWRDSPANTFKHR
37	Lst (pLTPro159 and wca-rfb	MGLKKVCLTVLCLIVFCFGIFYTFDRVNQGERNAVSLLKDKLFNEEGKPVNLI FCYTILQMKVAERIMAQHPGERFYVVLMSENRNEKYDYYFNQIKDKAERAYFF

SEQ ID NO.	Description	Sequence
	locus) from Neisseria gonorrhoeae 1291 (GenBank AAY41933)	YLPYGLNKSFNFIPTMAELKVKSMLLPKVKRIYLASLEKVSIAAFLSTYPDAE IKTFDDGTNNLIRESSYLGGEFAVNGAIKRNFARMMVGDWSIAKTRNASDEHY TIFKGLKNIMDDGRRKMTYLPLFDASELKAGDETGGTVRILLGSPDKEMKEIS EKAAKNFNIQYVAPHPRQTYGLSGVTALNSPYVIEDYILREIKKNPHTRYEIY TFFSGAALTMKDFPNVHVYALKPASLPEDYWLKPVYALFRQADIPILAFDDKN QSHGKSK
38	Exemplary nucleotide sequence encoding the AniA polypeptide (with signal peptide)	ATGAAACGCCAAGCATTAGCCGCAATGATTGCTTCCTTATTCGCATTGGCCGC CTGCGGTGGCGAACAAGCTGCTCAAGCCCCTGCCGAAACCCCTGCCGCTTCCG CAGAAGCCGCAAGTTCCGCCGCACAAGCTACCGCCGAAACGCCTGCAGGCGAA CTGCCCGTCATCGATGCGGTGACCACCCACGCTCCCGAAGTACCTCCCGCAAT CGACCGCGACTATCCTGCCAAAGTACGCGTAAAAATGGAAACCGTCGAAAAAA CCATGAAAATGGACGACGGGGTGGAATACCGCTACTGGACATTTGACGGCGAC GTTCCGGGCCGTATGATCCGCGTACGCGAAGGCGATACGGTTGAAGTCGAATT CTCCAACAATCCTTCTTCTACCGTTCCGCACAACGTCGACTTCCACGCCGCAA CCGGTCAGGGCGGCGGTGCAGCCGCGACCTTTACCGCCCCGGGCCGCACTTCC ACATTCAGCTTCAAAGCCCTGCAACCGGGCCTGTACATCTACCACTGCGCCGT CGCGCCGGTCGGTATGCACATCGCCAACGGTATGTACGGTCTGATTTTGGTCG AGCCTAAAGAAGGCCTGCCGAAAGTGGATAAAGAGTTCTACATCGTCCAAGGC GACTTCTACACCAAAGGCAAAAAAGGCGCGCAAGGCCTGCAACCGTTCGATAT GGACAAAGCCGTTGCCGAACAGCCTGAATACGTCGTATTCAACGGCCACGTAG GCGCTATCGCCGGCGATAACGCCCTGAAAGCCAAAGCAGGCGAAACCGTGCGT ATGTACGTCGGTAACGGCGGCCCGAACTTGGTGTCTTCCTTCCACGTCATCGG CGAAATCTTCGACAAAGTTTATGTTGAAGGCGGCAAACTGATTAACGAAAACG TACAAAGCACCATCGTGCCTGCCGGCGGTTCTGCCATCGTCGAATTCAAAGTC GACATCCCGGGCAACTACACTTTGGTCGACCACTCCATCTTCCGCGCATTCAA CAAAGGCGCGTTGGGGCAATTGAAAGTAGAGTAA
39	Exemplary nucleotide sequence encoding the MetQ polypeptide(with signal peptide)	ATGAAAACCTTCTTCAAAACCCTTTCCGCCGCCGCACTCGCGCTCATCCTCGC AGCCTGCGGCGGTCAAAAAGACAGCGCGCCCGCAGCCTCTGCCGCCGCCCCTT CTGCCGATAACGGCGCGGCGAAAAAAGAAATCGTCTTCGGCACGACCGTCGGC GACTTCGGCGATATGGTCAAAGAACAAATCCAAGCCGAGCTGGAGAAAAAAGG CTACACCGTCAAATTGGTCGAATTTACCGACTATGTGCGCCCGAATCTGGCAT TGGCGGAGGGCGAGTTGGACATCAACGTCTTCCAACACAAACCCTATCTTGAC GATTTCAAAAAAGAACACAACCTGGACATCACCGAAGCCTTCCAAGTGCCGAC CGCGCCTTTGGGACTGTATCCGGGCAAACTGAAATCGCTGGAAGAAGTCAAAG ACGGCAGCACCGTATCCGCGCCCAACGACCCGTCCAACTTCGCACGCGCCTTG GTGATGCTGAACGAACTGGGTTGGATCAAACTCAAAGACGGCATCAATCCGCT GACCGCATCCAAAGCCGATATCGCGGAAAACCTGAAAAACATCAAAATCGTCG AGCTTGAAGCCGCACAACTGCCGCGCAGCCGCGCCGACGTGGATTTTGCCGTC GTCAACGGCAACTACGCCATAAGCAGCGGCATGAAGCTGACCGAAGCCCTGTT CCAAGAGCCGAGCTTTGCCTATGTCAACTGGTCTGCCGTCAAAACCGCCGACA AAGACAGCCAATGGCTTAAAGACGTAACCGAGGCCTATAACTCCGACGCGTTC AAAGCCTACGCGCACAAACGCTTCGAGGGCTACAAATACCCTGCCGCATGGAA TGAAGGCGCAGCCAAATAA
40	Exemplary nucleotide sequence encoding the MsrA/B-AniA fusion polypeptide (with signal peptide)	ATGAAACGCCAAGCATTAGCCGCAATGATTGCTTCCTTATTCGCATTGGCCGC CTGCGGTGGCGAACAAGCTGCTCAAGCCCCTGCCGAAACCCCTGCCGCTTCCG CAGAAGCCGCAAGTTCCGCCGCACAAGCTACCGCCGAAACGCCTGCACCGCAC ACTTTATCCACGTTAAAAACCGCGGACAACCGCCCCGCCAGTGTTTATTTGAA AAAAGACAAACCGACGCTGATTAAATTTTGGGCGAGCTGGTGTCCTTTATGTT TGTCCGAATTGGGACAGGCCGAGAAATGGGCGCAAGATGCAAAATTCAGCTCC GCCAACCTGATTACCGTCGCCTCCCCCGGCTTTTTGCACGAGAAAAAAGACGG CGAGTTTCAAAAATGGTATGCCGGTTTGAACTACCCCAAGCTGCCCGTCGTTA CCGACAACGGCGGCACGATCGCCCAAAACCTGAATATCAGCGTTTATCCTTCT

SEQ ID NO.	Description	Sequence
		TGGGCGTTAATCGGTAAAGACGGCGACGTGCAGCGCATCGTCAAAGGCAGCAT CAACGAAGCGCAGGCATTGGCGTTAATCCGCAACCCGAATGCCGATTTGGGCA GTTTGAAACATTCGTAA
41	Exemplary nucleotide sequence encoding the NHBA-MetQ fusion polypeptide (with signal peptide)	ATGAAAACCTTCTTCAAAACCCTTTCCGCCGCCGCACTCGCGCTCATCCTCGC AGCCTGCGGCGGTCAAAAAGACAGCGCGCCCGCAGCCTCTGCCGCCGCCCCTT CTGCCGATAACGGCGCGGCGTCGCTTCCGGCCGAGATTCCGCTGATTCCCGTC AATCAGGCCGATACGCTGATTGTGGATGGGGAAGCGGTCAGCCTGACGGGGCA TTCCGGCAATATCTTCGCGCCCGAAGGGAATTACCGGTATCTGACTTACGGGG CGGAAAAATTGCCCGGCGGATCGTATGCCCTCCGTGTGCAAGGCGAACCGGCA AAAGGCGAAATGCTTGCGGGCACGGCCGTGTACAACGGCGAAGTGCTGCATTT CCATATGGAAAACGGCCGTCCGTACCCGTCCGGAGGCAGGTTTGCCGCAAAAG TCGATTTCGGCAGCAAATCTGTGGACGGCATTATCGACAGCAGCGATGATTTG CATATGGGTACGCAAAAATTCAAAGCCGCCATCGATGGAAACGGCTTTAAGGG GACTTGGACGGAAAATGGCGGCGGGGATGTTTCCGGAAGGTTTTACGGCCCGG CCGGCGAGGAAGTGGCGGGAAAATACAGCTATCGCCCGACAGATGCTGAAAAG GGCGGATTCGGCGTGTTTGCCGGCAAAAAAGATCGGGATTGA
42	Nucleotide sequence set of *E. coli* genome comprising pBAD promoter and exogenous polynucleotide encoding lpp attλ sites (duplicated upon insertion) are indicated by bold letters, polynucleotide encoding lpp is indicated by underlined letters; pBAD promoter is indicated by double underlined letters, FRT site is indicated by italic bold letters.	cagcaccgccgaaaggcgaaactcatcgctacatttttaccgttcacgcgctg gatatagaacgtattgatgtcgatgaaggtgccagcggcgcgatggtcgggtt taacgttcatttccactctctggcaagcgcctcgattactgcgatgtttagtt aatcactctgccagatggcgcaatgccatctggtatcacttaaaggtattaaa aacaacttttgtcttttaccttcccgtttcgctcaag**ttagtataaaaaag c**tgaacgagaaacgtaaaatgatataaatatcaatatattaaattagattttg cataaaaaacagactacataatactgtaaaacacaacatatatgcagtcacta tgaatcaactacttagatggtattagtgacctgtaacagagcattagcgcaag gtgatttttgtcttcttgcgctaatttttgtcatcaaacctgtcgcactcca gagaagcacaaagcctcgcaatccagtgcaaagctagcttcttcgtctgtttc tactggtattggcacaaacctgattccaatttgagcaaggctatgtgccatct cgatactcgttcttaactcaacagaagatgctttgtgcatacagcccctcgtt tattatttatctcctcagccagccgctgtgctttcagtggatttcggataaca gaaaggccgggaaatacccagcctcgctttgtaacggagtagagacgaaagtg attgcgcctacccggatattatcgtgaggatgcgtcatcgccattaattcact gatcagtgataagctgtcaaacatgagaattcgagctcggtacccgggttact tgcggtatttagtagccatgttgtccagacgctggttagcacgagctgcgtca tctttagcagcctgaacgtcggaacgcattgcgttcacgtcgttgctcagctg gtcaactttagcgttcagagtctgaacgtcagaagacagctgatcgattttag cgttgctggagcaacctgccagcagagtagaacccaggattaccgcgcccagt accagtttagtagctttcatatgaattcctccatccaaaaaaacgggtatgga gaaacagtagagagttgcgataaaaagcgtcaggtaggatccgctaatcttat ggataaaaatgctatggcatagcaaagtgtgacgccgtgcaaataatcaatgt ggacttttctgccgtgattatagacacttttgctacgcgtttttgtcatggcc ttggtcccgctttgttacagaatgcttttaataagcggggttaccggtttggt tagcgagaagagccagtaaaagacgcagtgacggcaatgtctgatgcaatatg gacaattggtttcttctctgaatggcggctgcaggcatgcaagcttggcactg gccacgcaaaaaggccatccgtcaggatggccttctgcttaatttgatgcctg gcagtttatggcgggcgtcctgcccgccaccctccgggccgttgcttcgcaac gttcaaatccgctcccggcggatttgtcctactcaggagagcgttcaccgaca aacaacagataaaacgaaaggcccagtctttcgactgagcctttcgttttatt tgatgcctggcagttccctactctcgcatggggagaccccacactaccatcgg gggccatcgatgcaggtggcacttttcggggaaatgtgcgcggaacccctatt tgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataacc ctgataaatgcttcaataatctagtggatcaagagacaggatgaggatcgttt cgcacatatgaatatcctcctt***agttcctattccgaagttcctattctctaga aagtataggaacttc***gaagcagctccagcctacacgggactctggggttcga

SEQ ID NO.	Description	Sequence
		taccgtcaaaacttcatttttaatttttgcggccgcaagatccggccacgatg cgtccggcgtagaggatctgaagatcagcagttcaacctgttgatagtacgta ctaagctctcatgtttcacgtactaagctctcatgtttaacgtactaagctct catgtttaacgaactaaaccctcatggctaacgtactaagctctcatggctaa cgtactaagctctcatgtttcacgtactaagctctcatgtttgaacaataaaa ttaatataaatcagcaacttaaatagcctctaaggttttaagttttataagaa aaaaaagaatatataaggcttttaaagcttttaaggtttaacggttgtggaca acaagccagggatgtaacgcactgagaagcccttagagcctctcaaagcaatt ttgagtgacacaggaacacttaacggctgacatgggaattagccatggcatca cagtatcgtgatgacagaggcagggagtgggacaaaattgaaatcaaataatg atttttattttgactgatagtgacctgttcgttgcaacaaattgataagcaatg ctttttttataatgccaac**ttagtataaaaaagc**aggcttcaacggattcattt ttctatttcatagcccggagcaacctgtgaacacattttcagtttcccgtctg gcgctggcattggcttttggcgtgacgctgaccgcctgtagctcaaccccgcc cgatcaacgtccttctgatcaaaccgcgcctggtacctcttctcgcccgatt
43	Exemplary amino acid sequence of Lpp	MKATKLVLGAVILGSTLLAGCSSNAKIDQLSSDVQTLNAKVDQLSNDVNAMRS DVQAAKDDAARANQRLDNMATKYRK
44	Exemplary nucleotide sequence encoding the AniA polypeptide (without signal peptide)	TGCGGTGGCGAACAAGCTGCTCAAGCCCCTGCCGAAACCCCTGCCGCTTCCGC AGAAGCCGCAAGTTCCGCCGCACAAGCTACCGCCGAAACGCCTGCAGGCGAAC TGCCCGTCATCGATGCGGTGACCACCCACGCTCCCGAAGTACCTCCCGCAATC GACCGCGACTATCCTGCCAAAGTACGCGTAAAAATGGAAACCGTCGAAAAAAC CATGAAAATGGACGACGGGGTGGAATACCGCTACTGGACATTTGACGGCGACG TTCCGGGCCGTATGATCCGCGTACGCGAAGGCGATACGGTTGAAGTCGAATTC TCCAACAATCCTTCTTCTACCGTTCCGCACAACGTCGACTTCCACGCCGCAAC CGGTCAGGGCGGCGGTGCAGCCGCGACCTTTACCGCCCCGGGCCGCACTTCCA CATTCAGCTTCAAAGCCCTGCAACCGGGCCTGTACATCTACCACTGCGCCGTC GCGCCGGTCGGTATGCACATCGCCAACGGTATGTACGGTCTGATTTTGGTCGA GCCTAAAGAAGGCCTGCCGAAAGTGGATAAAGAGTTCTACATCGTCCAAGGCG ACTTCTACACCAAAGGCAAAAAAGGCGCGCAAGGCCTGCAACCGTTCGATATG GACAAAGCCGTTGCCGAACAGCCTGAATACGTCGTATTCAACGGCCACGTAGG CGCTATCGCCGGCGATAACGCCCTGAAAGCCAAAGCAGGCGAAACCGTGCGTA TGTACGTCGGTAACGGCGGCCCGAACTTGGTGTCTTCCTTCCACGTCATCGGC GAAATCTTCGACAAAGTTTATGTTGAAGGCGGCAAACTGATTAACGAAAACGT ACAAAGCACCATCGTGCCTGCCGGCGGTTCTGCCATCGTCGAATTCAAAGTCG ACATCCCGGGCAACTACACTTTGGTCGACCACTCCATCTTCCGCGCATTCAAC AAAGGCGCGTTGGGGCAATTGAAAGTAGAGTAA
45	Exemplary nucleotide sequence encoding the MetQ polypeptide (without signal peptide)	TGCGGCGGTCAAAAAGACAGCGCGCCCGCAGCCTCTGCCGCCGCCCCTTCTGC CGATAACGGCGCGGCGAAAAAAGAAATCGTCTTCGGCACGACCGTCGGCGACT TCGGCGATATGGTCAAAGAACAAATCCAAGCCGAGCTGGAGAAAAAAGGCTAC ACCGTCAAATTGGTCGAATTTACCGACTATGTGCGCCCGAATCTGGCATTGGC GGAGGGCGAGTTGGACATCAACGTCTTCCAACACAAACCCTATCTTGACGATT TCAAAAAAGAACACAACCTGGACATCACCGAAGCCTTCCAAGTGCCGACCGCG CCTTTGGGACTGTATCCGGGCAAACTGAAATCGCTGGAAGAAGTCAAAGACGG CAGCACCGTATCCGCGCCCAACGACCCGTCCAACTTCGCACGCGCCTTGGTGA TGCTGAACGAACTGGGTTGGATCAAACTCAAAGACGGCATCAATCCGCTGACC GCATCCAAAGCCGATATCGCGGAAAACCTGAAAAACATCAAAATCGTCGAGCT TGAAGCCGCACAACTGCCGCGCAGCCGCGCCGACGTGGATTTTGCCGTCGTCA ACGGCAACTACGCCATAAGCAGCGGCATGAAGCTGACCGAAGCCCTGTTCCAA GAGCCGAGCTTTGCCTATGTCAACTGGTCTGCCGTCAAAACCGCCGACAAAGA CAGCCAATGGCTTAAAGACGTAACCGAGGCCTATAACTCCGACGCGTTCAAAG

SEQ ID NO.	Description	Sequence
		CCTACGCGCACAAACGCTTCGAGGGCTACAAATACCCTGCCGCATGGAATGAA GGCGCAGCCAAATAA
46	Exemplary nucleotide sequence encoding the MsrA/B polypeptide	CCGCACACTTTATCCACGTTAAAAACCGCGGACAACCGCCCCGCCAGTGTTTA TTTGAAAAAAGACAAACCGACGCTGATTAAATTTTGGGCGAGCTGGTGTCCTT TATGTTTGTCCGAATTGGGACAGGCCGAGAAATGGGCGCAAGATGCAAAATTC AGCTCCGCCAACCTGATTACCGTCGCCTCCCCCGGCTTTTTGCACGAGAAAAA AGACGGCGAGTTTCAAAAATGGTATGCCGGTTTGAACTACCCCAAGCTGCCCG TCGTTACCGACAACGGCGGCACGATCGCCCAAAACCTGAATATCAGCGTTTAT CCTTCTTGGGCGTTAATCGGTAAAGACGGCGACGTGCAGCGCATCGTCAAAGG CAGCATCAACGAAGCGCAGGCATTGGCGTTAATCCGCAACCCGAATGCCGATT TGGGCAGTTTGAAACATTCGTAA
47	Exemplary nucleotide sequence encoding the MsrA/B-AniA fusion polypeptide (without signal peptide)	TGCGGTGGCGAACAAGCTGCTCAAGCCCCTGCCGAAACCCCTGCCGCTTCCGC AGAAGCCGCAAGTTCCGCCGCACAAGCTACCGCCGAAACGCCTGCACCGCACA CTTTATCCACGTTAAAAACCGCGGACAACCGCCCCGCCAGTGTTTATTTGAAA AAAGACAAACCGACGCTGATTAAATTTTGGGCGAGCTGGTGTCCTTTATGTTT GTCCGAATTGGGACAGGCCGAGAAATGGGCGCAAGATGCAAAATTCAGCTCCG CCAACCTGATTACCGTCGCCTCCCCCGGCTTTTTGCACGAGAAAAAAGACGGC GAGTTTCAAAAATGGTATGCCGGTTTGAACTACCCCAAGCTGCCCGTCGTTAC CGACAACGGCGGCACGATCGCCCAAAACCTGAATATCAGCGTTTATCCTTCTT GGGCGTTAATCGGTAAAGACGGCGACGTGCAGCGCATCGTCAAAGGCAGCATC AACGAAGCGCAGGCATTGGCGTTAATCCGCAACCCGAATGCCGATTTGGGCAG TTTGAAACATTCGTAA
48	Exemplary nucleotide sequence encoding the NHBA polypeptide	TCGCTTCCGGCCGAGATTCCGCTGATTCCCGTCAATCAGGCCGATACGCTGAT TGTGGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGC CCGAAGGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGA TCGTATGCCCTCCGTGTGCAAGGCGAACCGGCAAAAGGCGAAATGCTTGCGGG CACGGCCGTGTACAACGGCGAAGTGCTGCATTTCCATATGGAAAACGGCCGTC CGTACCCGTCCGGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGCAGCAAATCT GTGGACGGCATTATCGACAGCAGCGATGATTTGCATATGGGTACGCAAAAATT CAAAGCCGCCATCGATGGAAACGGCTTTAAGGGGACTTGGACGGAAAATGGCG GCGGGGATGTTTCCGGAAGGTTTTACGGCCCGGCCGGCGAGGAAGTGGCGGGA AAATACAGCTATCGCCCGACAGATGCTGAAAAGGGCGGATTCGGCGTGTTTGC CGGCAAAAAAGATCGGGATTGA
49	Exemplary nucleotide sequence encoding the NHBA-MetQ fusion polypeptide (without signal peptide)	TGCGGCGGTCAAAAAGACAGCGCGCCCGCAGCCTCTGCCGCCGCCCCTTCTGC CGATAACGGCGCGGCGTCGCTTCCGGCCGAGATTCCGCTGATTCCCGTCAATC AGGCCGATACGCTGATTGTGGATGGGGAAGCGGTCAGCCTGACGGGGCATTCC GGCAATATCTTCGCGCCCGAAGGGAATTACCGGTATCTGACTTACGGGGCGGA AAAATTGCCCGGCGGATCGTATGCCCTCCGTGTGCAAGGCGAACCGGCAAAAG GCGAAATGCTTGCGGGCACGGCCGTGTACAACGGCGAAGTGCTGCATTTCCAT ATGGAAAACGGCCGTCCGTACCCGTCCGGAGGCAGGTTTGCCGCAAAAGTCGA TTTCGGCAGCAAATCTGTGGACGGCATTATCGACAGCAGCGATGATTTGCATA TGGGTACGCAAAAATTCAAAGCCGCCATCGATGGAAACGGCTTTAAGGGGACT TGGACGGAAAATGGCGGCGGGGATGTTTCCGGAAGGTTTTACGGCCCGGCCGG CGAGGAAGTGGCGGGAAAATACAGCTATCGCCCGACAGATGCTGAAAAGGGCG GATTCGGCGTGTTTGCCGGCAAAAAAGATCGGGATTGA
50	Full length AniA polypeptide amino acid sequence	CGGEQAAQAPAETPAASAEAASSAAQATAETPAGELPVIDAVTTHAPEVPPAI DRDYPAKVRVKMETVEKTMKMDDGVEYRYWTFDGDVPGRMIRVREGDTVEVEF SNNPSSTVPHNVDFHAATGQGGGAAATFTAPGRTSTFSFKALQPGLYIYHCAV APVGMHIANGMYGLILVEPKEGLPKVDKEFYIVQGDFYTKGKKGAQGLQPFDM DKAVAEQPEYVVFNGHVGAIAGDNALKAKAGETVRMYVGNGGPNLVSSFHVIG

SEQ ID NO.	Description	Sequence
	(without signal peptide)	EIFDKVYVEGGKLINENVQSTIVPAGGSAIVEFKVDIPGNYTLVDHSIFRAFN KGALGQLKVEGAENPEIMTQKLSDTAYAGSGAASAPAASAPAASAPAASAPAA SAPAASASEKSVY
51	Full length MsrA/B polypeptide (without signal peptide)	CSPKIVDAGTATVPHTLSTLKTADNRPASVYLKKDKPTLIKFWASWCPLCLSE LGQAEKWAQDAKFSSANLITVASPGFLHEKKDGEFQKWYAGLNYPKLPVVTDN GGTIAQNLNISVYPSWALIGKDGDVQRIVKGSINEAQALALIRNPNADLGSLK HSFYKPDTQKKDSAIMNTRTIYLAGGCFWGLEAYFQRIDGVVDAVSGYANGNT ENPSYEDVSYRHTGHAETVKVTYDADKLSLDDILQYYFRVVDPTSLNKQGNDT GTQYRSGVYYTDPAEKAVIAAALKREQQKYQLPLVVENEPLKNFYDAEEYHQD YLIKNPNGYCHIDIRKADEPLPGKTKAAPQGKGFDAATYKKPSDAELKRTLTE EQYQVTQNSATEYAFSHEYDHLFKPGIYVDVVSGEPLFSSADKYDSGCGWPSF TRPIDAKSVTEHDDFSFNMRRTEVRSRAADSHLGHVFPDGPRDKGGLRYCING ASLKFIPLEQMDAAGYGALKGKVK
52	Full length NHBA polypeptide (without signal peptide)	CGGGGGGSPDVKSADTPSKPAAPVVAENAGEGVLPKEKKDEEAAGGAPQADTQ DATAGEGSQDMAAVSAENTGNGGAATTDNPKNEDAGAQNDMPQNAAESANQTG NNQPAGSSDSAPASNPAPANGGSDFGRTNVGNSVVIDGPSQNITLTHCKGDPC NGDNLLDEEAPPKSEFESLSDEEKIKKYKKDGEKFTGLVAIKVENNGLNKYTI IYQAQPTRSARSRRSLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGN YRYLTYGAEKLPGGSYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPS GGRFAAKVDFGSKSVDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDV SGRFYGPAGEEVAGKYSYRPTDAEKGGFGVFAGKKDRD
53	KdsB (pLTPro231 and pLTPro302) from Escherichia coli K12 (GenBank WP_000011603)	MSFVVIIPARYASTRLPGKPLVDINGKPMIVHVLERARESGAERIIVATDHED VARAVEAAGGEVCMTRADHQSGTERLAEVVEKCAFSDDTVIVNVQGDEPMIPA TIIRQVADNLAQRQVGMATLAVPIHNAEEAFNPNAVKVVLDAEGYALYFSRAT IPWDRDRFAEGLETVGDNFLRHLGIYGYRAGFIRRYVNWQPSPLEHIEMLEQL RVLWYGEKIHVAVAQEVPGTGVDTPEDLERVRAEMR
54	Exemplary nucleotide sequence encoding KdsB	ATGAGTTTTGTGGTCATTATTCCCGCGCGCTACGCGTCCACGCGTCTGCCCGG TAAACCATTGGTTGATATTAACGGCAAACCCATGATTGTTCATGTTCTTGAAC GCGCGCGTGAATCAGGTGCCGAGCGCATCATCGTGGCAACCGATCATGAGGAT GTTGCCCGCGCCGTTGAAGCCGCTGGCGGTGAAGTATGTATGACGCGCGCCGA TCATCAGTCAGGAACAGAACGTCTGGCGGAAGTTGTCGAAAAATGCGCATTCA GCGACGACACGGTGATCGTTAATGTGCAGGGTGATGAACCGATGATCCCTGCG ACAATCATTCGTCAGGTTGCTGATAACCTCGCTCAGCGTCAGGTGGGTATGGC GACTCTGGCGGTGCCAATCCACAATGCGGAAGAAGCGTTTAACCCGAATGCGG TGAAAGTGGTTCTCGACGCTGAAGGGTATGCACTGTACTTCTCTCGCGCCACC ATTCCTTGGGATCGTGATCGTTTTGCAGAAGGCCTTGAAACCGTTGGCGATAA CTTCCTGCGTCATCTTGGTATTTATGGCTACCGTGCAGGCTTTATCCGTCGTT ACGTCAACTGGCAGCCAAGTCCGTTAGAACACATCGAAATGTTAGAGCAGCTT CGTGTTCTGTGGTACGGCGAAAAAATCCATGTTGCTGTTGCTCAGGAAGTTCC TGGCACAGGTGTGGATACCCCTGAAGATCTTGAGCGCGTTCGCGCTGAAATGC GCTAG
55	Region containing the ECA promoter	tttgtcttatgccaaaaacgccacgtgtttacgtggcgttttgcttttatatc tgtaatcttaatgccgcgctggcgatgttaggaaaattcctggaatttgctgg catgttatgcaatttgcatatcaaatggttaatttttgcacaggactggtggg tttggaacggactttcccttctgaataaaggtcttcgtggttatacttctgct aataattttctctgagagcatgcattg

SEQ ID NO.	Description	Sequence
56	Inserted sequence containing the O antigen promoter	ctgaccggatgtaacggttgataagaaaattataacggcagtgaaaattcgca gcaaaagtaatttgttgcgaatcttcctgccgttgttttatataaaccatcag aataacaacgagttagcagtagggttttattcaaagttttccaggattttcct tgtttccagagcggattggtaagacaattagcgtttgaatttttcgggtttag cgcgagtgggtaacgctcgtcacatcataggcatgcatgcagtgctctggtag ctgtaaagccaggggcggtagcgtgcattaatacctctattaatcaaactgag agccgcttatttcacagcatgctctgaagtaatatggaataaattaa
57	Exemplary Met1 polypeptide amino acid sequence (without signal peptide)	CGGQKDSAPAASAAAPSADNGAAKKEIVFGTTVGDFGDMVKEQIQAELEKKGY TVKLVEFTDYVRPNLALAEGELDINVFQHKPYLDDFKKEHNLDITEAFQVPT
58	Exemplary Met2 polypeptide amino acid sequence (without signal peptide)	PTAPLGLYPGKLKSLEEVKDGSTVSAPNDPSNFARALVMLNELGWIKLKDGIN PLTASKADIAENLKNIKIVELEAAQLPRSRADVDFAVVNGNYAISSGMKLTEA LFQEPSFAYVNWSAVKTADKDSQWLKDVTEAYNSDAFKAYAHKRFEGYKYPAA WNEGAAK
60	Exemplary Met1_cNHBA fusion polypeptide (without signal peptide)	CGGQKDSAPAASAAAPSADNGAAKKEIVFGTTVGDFGDMVKEQIQAELEKKGY TVKLVEFTDYVRPNLALAEGELDINVFQHKPYLDDFKKEHNLDITEAFQVPTG GGGSLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKL PGGSYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFG SKSVDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEE VAGKYSYRPTDAEKGGFGVFAGKKDRD
61	Exemplary Met2_cNHBA fusion polypeptide (without signal peptide)	PTAPLGLYPGKLKSLEEVKDGSTVSAPNDPSNFARALVMLNELGWIKLKDGIN PLTASKADIAENLKNIKIVELEAAQLPRSRADVDFAVVNGNYAISSGMKLTEA LFQEPSFAYVNWSAVKTADKDSQWLKDVTEAYNSDAFKAYAHKRFEGYKYPAA WNEGAAKGGGGSLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRY LTYGAEKLPGGSYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGR FAAKVDFGSKSVDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGR FYGPAGEEVAGKYSYRPTDAEKGGFGVFAGKKDRD
62	Exemplary MetQ_cNHBA fusion polypeptide (without signal peptide)	CGGQKDSAPAASAAAPSADNGAAKKEIVFGTTVGDFGDMVKEQIQAELEKKGY TVKLVEFTDYVRPNLALAEGELDINVFQHKPYLDDFKKEHNLDITEAFQVPTA PLGLYPGKLKSLEEVKDGSTVSAPNDPSNFARALVMLNELGWIKLKDGINPLT ASKADIAENLKNIKIVELEAAQLPRSRADVDFAVVNGNYAISSGMKLTEALFQ EPSFAYVNWSAVKTADKDSQWLKDVTEAYNSDAFKAYAHKRFEGYKYPAAWNE GAAKGGGGSLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTY GAEKLPGGSYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAA KVDFGSKSVDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYG PAGEEVAGKYSYRPTDAEKGGFGVFAGKKDRD
63	Exemplary cNHBA_Met1 fusion polypeptide (without signal peptide)	SLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPGG SYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFGSKS VDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEEVAG KYSYRPTDAEKGGFGVFAGKKDRDGGGGCGGQKDSAPAASAAAPSADNGAAKK EIVFGTTVGDFGDMVKEQIQAELEKKGYTVKLVEFTDYVRPNLALAEGELDIN VFQHKPYLDDFKKEHNLDITEAFQVPT
64	Exemplary cNHBA_Met2	SLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPGG SYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFGSKS

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	fusion polypeptide (without signal peptide)	VDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEEVAG KYSYRPTDAEKGGFGVFAGKKDRDGGGGPTAPLGLYPGKLKSLEEVKDGSTVS APNDPSNFARALVMLNELGWIKLKDGINPLTASKADIAENLKNIKIVELEAAQ LPRSRADVDFAVVNGNYAISSGMKLTEALFQEPSFAYVNWSAVKTADKDSQWL KDVTEAYNSDAFKAYAHKRFEGYKYPAAWNEGAAK
65	Exemplary cNHBA_MetQ fusion polypeptide (without signal peptide)	SLPAEIPLIPVNQADTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPGG SYALRVQGEPAKGEMLAGTAVYNGEVLHFHMENGRPYPSGGRFAAKVDFGSKS VDGIIDSSDDLHMGTQKFKAAIDGNGFKGTWTENGGGDVSGRFYGPAGEEVAG KYSYRPTDAEKGGFGVFAGKKDRDGGGGCGGQKDSAPAASAAAPSADNGAAKK EIVFGTTVGDFGDMVKEQIQAELEKKGYTVKLVEFTDYVRPNLALAEGELDIN VFQHKPYLDDFKKEHNLDITEAFQVPTAPLGLYPGKLKSLEEVKDGSTVSAPN DPSNFARALVMLNELGWIKLKDGINPLTASKADIAENLKNIKIVELEAAQLPR SRADVDFAVVNGNYAISSGMKLTEALFQEPSFAYVNWSAVKTADKDSQWLKDV TEAYNSDAFKAYAHKRFEGYKYPAAWNEGAAK
66	pLTPro231 sequences	ATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCG TAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATG TTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTT GACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATT TCACACAGGAAACAGATGAAACGCCAAGCATTAGCCGCAATGATTGCTTCCTT ATTCGCATTGGCCGCCTGCGGTGGCGAACAAGCTGCTCAAGCCCCTGCCGAAA CCCCTGCCGCTTCCGCAGAAGCCGCAAGTTCCGCCGCACAAGCTACCGCCGAA ACGCCTGCACCGCACACTTTATCCACGTTAAAAACCGCGGACAACCGCCCCGC CAGTGTTTATTTGAAAAAAGACAAACCGACGCTGATTAAATTTTGGGCGAGCT GGTGTCCTTTATGTTTGTCCGAATTGGGACAGGCCGAGAAATGGGCGCAAGAT GCAAAATTCAGCTCCGCCAACCTGATTACCGTCGCCTCCCCCGGCTTTTTGCA CGAGAAAAAAGACGGCGAGTTTCAAAAATGGTATGCCGGTTTGAACTACCCCA AGCTGCCCGTCGTTACCGACAACGGCGGCACGATCGCCCAAAACCTGAATATC AGCGTTTATCCTTCTTGGGCGTTAATCGGTAAAGACGGCGACGTGCAGCGCAT CGTCAAAGGCAGCATCAACGAAGCGCAGGCATTGGCGTTAATCCGCAACCCGA ATGCCGATTTGGGCAGTTTGAAACATTCGTAAGAGCTGAATTCGAGCTCAGGA GGAGGGATATGAGTTTTGTGGTCATTATTCCCGCGCGCTACGCGTCCACGCGT CTGCCCGGTAAACCATTGGTTGATATTAACGGCAAACCCATGATTGTTCATGT TCTTGAACGCGCGCGTGAATCAGGTGCCGAGCGCATCATCGTGGCAACCGATC ATGAGGATGTTGCCCGCGCCGTTGAAGCCGCTGGCGGTGAAGTATGTATGACG CGCGCCGATCATCAGTCAGGAACAGAACGTCTGGCGGAAGTTGTCGAAAAATG CGCATTCAGCGACGACACGGTGATCGTTAATGTGCAGGGTGATGAACCGATGA TCCCTGCGACAATCATTCGTCAGGTTGCTGATAACCTCGCTCAGCGTCAGGTG GGTATGGCGACTCTGGCGGTGCCAATCCACAATGCGGAAGAAGCGTTTAACCC GAATGCGGTGAAAGTGGTTCTCGACGCTGAAGGGTATGCACTGTACTTCTCTC GCGCCACCATTCCTTGGGATCGTGATCGTTTTGCAGAAGGCCTTGAAACCGTT GGCGATAACTTCCTGCGTCATCTTGGTATTTATGGCTACCGTGCAGGCTTTAT CCGTCGTTACGTCAACTGGCAGCCAAGTCCGTTAGAACACATCGAAATGTTAG AGCAGCTTCGTGTTCTGTGGTACGGCGAAAAAATCCATGTTGCTGTTGCTCAG GAAGTTCCTGGCACAGGTGTGGATACCCCTGAAGATCTTGAGCGCGTTCGCGC TGAAATGCGCTAGGCTAGCAGCTCAGGAGGAGATAACAGATGAAAACCTTCTT CAAAACCCTTTCCGCCGCCGCACTCGCGCTCATCCTCGCAGCCTGCGGCGGTC AAAAAGACAGCGCGCCCGCAGCCTCTGCCGCCGCCCCTTCTGCCGATAACGGC GCGGCGAAAAAAGAAATCGTCTTCGGCACGACCGTCGGCGACTTCGGCGATAT GGTCAAAGAACAAATCCAAGCCGAGCTGGAGAAAAAAGGCTACACCGTCAAAT TGGTCGAATTTACCGACTATGTGCGCCCGAATCTGGCATTGGCGGAGGGCGAG TTGGACATCAACGTCTTCCAACACAAACCCTATCTTGACGATTTCAAAAAAGA ACACAACCTGGACATCACCGAAGCCTTCCAAGTGCCGACCGCGCCTTTGGGAC TGTATCCGGGCAAACTGAAATCGCTGGAAGAAGTCAAAGACGGCAGCACCGTA

SEQ ID NO.	Description	Sequence
		TCCGCGCCCAACGACCCGTCCAACTTCGCACGCGCCTTGGTGATGCTGAACGA ACTGGGTTGGATCAAACTCAAAGACGGCATCAATCCGCTGACCGCATCCAAAG CCGATATCGCGGAAAACCTGAAAAACATCAAAATCGTCGAGCTTGAAGCCGCA CAACTGCCGCGCAGCCGCGCCGACGTGGATTTTGCCGTCGTCAACGGCAACTA CGCCATAAGCAGCGGCATGAAGCTGACCGAAGCCCTGTTCCAAGAGCCGAGCT TTGCCTATGTCAACTGGTCTGCCGTCAAAACCGCCGACAAAGACAGCCAATGG CTTAAAGACGTAACCGAGGCCTATAACTCCGACGCGTTCAAAGCCTACGCGCA CAAACGCTTCGAGGGCTACAAATACCCTGCCGCATGGAATGAAGGCGCAGCCA AATAAAAGCTTAGGAGGAGATAACAGATGAAACGCCAAGCATTAGCCGCAATG ATTGCTTCCTTATTCGCATTGGCCGCCTGCGGTGGCGAACAAGCTGCTCAAGC CCCTGCCGAAACCCCTGCCGCTTCCGCAGAAGCCGCAAGTTCCGCCGCACAAG CTACCGCCGAAACGCCTGCAGGCGAACTGCCCGTCATCGATGCGGTGACCACC CACGCTCCCGAAGTACCTCCCGCAATCGACCGCGACTATCCTGCCAAAGTACG CGTAAAAATGGAAACCGTCGAAAAAACCATGAAAATGGACGACGGGGTGGAAT ACCGCTACTGGACATTTGACGGCGACGTTCCGGGCCGTATGATCCGCGTACGC GAAGGCGATACGGTTGAAGTCGAATTCTCCAACAATCCTTCTTCTACCGTTCC GCACAACGTCGACTTCCACGCCGCAACCGGTCAGGGCGGCGGTGCAGCCGCGA CCTTTACCGCCCCGGGCCGCACTTCCACATTCAGCTTCAAAGCCCTGCAACCG GGCCTGTACATCTACCACTGCGCCGTCGCGCCGGTCGGTATGCACATCGCCAA CGGTATGTACGGTCTGATTTTGGTCGAGCCTAAAGAAGGCCTGCCGAAAGTGG ATAAAGAGTTCTACATCGTCCAAGGCGACTTCTACACCAAAGGCAAAAAAGGC GCGCAAGGCCTGCAACCGTTCGATATGGACAAAGCCGTTGCCGAACAGCCTGA ATACGTCGTATTCAACGGCCACGTAGGCGCTATCGCCGGCGATAACGCCCTGA AAGCCAAAGCAGGCGAAACCGTGCGTATGTACGTCGGTAACGGCGGCCCGAAC TTGGTGTCTTCCTTCCACGTCATCGGCGAAATCTTCGACAAAGTTTATGTTGA AGGCGGCAAACTGATTAACGAAAACGTACAAAGCACCATCGTGCCTGCCGGCG GTTCTGCCATCGTCGAATTCAAAGTCGACATCCCGGGCAACTACACTTTGGTC GACCACTCCATCTTCCGCGCATTCAACAAAGGCGCGTTGGGGCAATTGAAAGT AGAGTAAGGTACCAGGAGGAGATAACAGATGAAAACCTTCTTCAAAACCCTTT CCGCCGCCGCACTCGCGCTCATCCTCGCAGCCTGCGGCGGTCAAAAAGACAGC GCGCCCGCAGCCTCTGCCGCCGCCCCTTCTGCCGATAACGGCGCGGCGTCGCT TCCGGCCGAGATTCCGCTGATTCCCGTCAATCAGGCCGATACGCTGATTGTGG ATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAA GGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGATCGTA TGCCCTCCGTGTGCAAGGCGAACCGGCAAAAGGCGAAATGCTTGCGGGCACGG CCGTGTACAACGGCGAAGTGCTGCATTTCCATATGGAAAACGGCCGTCCGTAC CCGTCCGGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGCAGCAAATCTGTGGA CGGCATTATCGACAGCAGCGATGATTTGCATATGGGTACGCAAAAATTCAAAG CCGCCATCGATGGAAACGGCTTTAAGGGGACTTGGACGGAAAATGGCGGCGGG GATGTTTCCGGAAGGTTTTACGGCCCGGCCGGCGAGGAAGTGGCGGGAAAATA CAGCTATCGCCCGACAGATGCTGAAAAGGGCGGATTCGGCGTGTTTGCCGGCA AAAAAGATCGGGATTGAGGATCCTGGCTGTTTTGGCGGATGAGAGAAGAAATT CGTCGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCGTCA GGATGGCCTTCTGCTTAATTTGATGCCTGGCAGTTTATGGCGGGCGACGAATC AGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCT AAATATACATTCAAATATGTATCCGCTCATGAGCTTCACGCTGCCGCAAGCAC TCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCA GAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGG AAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGA TAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGG GGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCT TGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGA TGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGC CGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCT GCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTT

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		GTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCG GCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGAT CTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGC AATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAG CGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGAT CAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGC CAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCG ATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATC GACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTAC CCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGC TTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTT GACGAGTTCTTCTGACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATA ATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGAC CCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAAT CTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGG ATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAG ATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAA CTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTA CCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAG CTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAG AAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTA TCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGT GATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTT TTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTT ATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCG CTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCGAA CGCCAGCAAGACGTAGCCCAGCGCGTCGGCCAATTCGCGCTAACTTACATTAA TTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCA GGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACC GCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAG GCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTT CGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGAC TCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCAT CGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGG ACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGA GTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAA TGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCA CGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTC TGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCAC AGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGC GTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGT TCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAAT CGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGC CAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATG TAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAAC GTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCAT ACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGA CTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCACCATTCGAT GGTGTCAACGTAAATGCATGCCGCTTCGCCTTCGCGCGCGAATTGGCCGCCAT GCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGA

SEQ ID NO.	Description	Sequence
		CGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCG ATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGC TGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGG CGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCT CTCAAGGGCATCGGTCGAGATCCGGGCTTATCGACTGCACGGTGCACCA
67	pLTPro302 sequences	GCGCAGCGAGTCAGTGAGCGAGGAAGCCGAACGCCAGCAAGACGTAGCCCAGC GCGTCGGCCAATTCGCGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGC TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCG CGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAG TGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCA GCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTG GTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTAC CGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGC CCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCA TTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTC CCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAG CCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATT TGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTC ATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATA ACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCC AGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCAC CGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGC TGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGC GCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCC CGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCG CTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACG CGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGT TACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATG CCATACCGCGAAAGGTTTTGCACCATTCGATGGTGTCAACGTAAATGCATGCC GCTTCGCCTTCGCGCGCGAATTGGCCGCCATGCCGGCGATAATGGCCTGCTTC TCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTG CAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAA AGCGGTCCTCGCCGAAAATGACCCAGAGCCTGTTGAGTAATAGTCAAAAGCCT CCGGTCGGAGGCTTTTGACTTTCTGCTTACTGAATTTCGGTGGTGCCGTTACT AGTCTAGCGCATTTCAGCGCGAACGCGCTCAAGATCTTCAGGGGTATCCACAC CTGTGCCAGGAACTTCCTGAGCAACAGCAACATGGATTTTTTCGCCGTACCAC AGAACACGAAGCTGCTCTAACATTTCGATGTGTTCTAACGGACTTGGCTGCCA GTTGACGTAACGACGGATAAAGCCTGCACGGTAGCCATAAATACCAAGATGAC GCAGGAAGTTATCGCCAACGGTTTCAAGGCCTTCTGCAAAACGATCACGATCC CAAGGAATGGTGGCGCGAGAGAAGTACAGTGCATACCCTTCAGCGTCGAGAAC CACTTTCACCGCATTCGGGTTAAACGCTTCTTCCGCATTGTGGATTGGCACCG CCAGAGTCGCCATACCCACCTGACGCTGAGCGAGGTTATCAGCAACCTGACGA ATGATTGTCGCAGGGATCATCGGTTCATCACCCTGCACATTAACGATCACCGT GTCGTCGCTGAATGCGCATTTTTCGACAACTTCCGCCAGACGTTCTGTTCCTG ACTGATGATCGGCGCGCGTCATACATACTTCACCGCCAGCGGCTTCAACGGCG CGGGCAACATCCTCATGATCGGTTGCCACGATGATGCGCTCGGCACCTGATTC ACGCGCGCGTTCAAGAACATGAACAATCATGGGTTTGCCGTTAATATCAACCA ATGGTTTACCGGGCAGACGCGTGGACGCGTAGCGCGCGGGAATAATGACCACA AAACTCATATCCCTCCTCCTGAGCTCGAATTCAGCTCCTAGTTTACTCTACTT TCAATTGCCCCAACGCGCCTTTGTTGAATGCGCGGAAGATGGAGTGGTCGACC AAAGTGTAGTTGCCCGGGATGTCGACTTTGAATTCGACGATGGCAGAACCGCC GGCAGGCACGATGGTGCTTTGTACGTTTTCGTTAATCAGTTTGCCGCCTTCAA CATAAACTTTGTCGAAGATTTCGCCGATGACGTGGAAGGAAGACACCAAGTTC GGGCCGCCGTTACCGACGTACATACGCACGGTTTCGCCTGCTTTGGCTTTCAG

SEQ ID NO.	Description	Sequence
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SEQ ID NO.	Description	Sequence
		GTGGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGCC CGAAGGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGAT CGTATGCCCTCCGTGTGCAAGGCGAACCGGCAAAAGGCGAAATGCTTGCGGGC ACGGCCGTGTACAACGGCGAAGTGCTGCATTTCCATATGGAAAACGGCCGTCC GTACCCGTCCGGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGCAGCAAATCTG TGGACGGCATTATCGACAGCAGCGATGATTTGCATATGGGTACGCAAAAATTC AAAGCCGCCATCGATGGAAACGGCTTTAAGGGGACTTGGACGGAAAATGGCGG CGGGGATGTTTCCGGAAGGTTTTACGGCCCGGCCGGCGAGGAAGTGGCGGGAA AATACAGCTATCGCCCGACAGATGCTGAAAAGGGCGGATTCGGCGTGTTTGCC GGCAAAAAAGATCGGGATTGAAAGCTTGGCTGTTTTGGCGGATGAGAGAAGAA ATTCGTCGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCG TCAGGATGGCCTTCTGCTTAATTTGATGCCTGGCAGTTTATGGCGGGCGACGA ATCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTT TCTAAATATACATTCAAATATGTATCCGCTCATGAGCTTCACGCTGCCGCAAG CACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCC GCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAA GGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGG CGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGC TGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTT TCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACA GGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCC GGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCG GCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTT TTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGC GCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACG TTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAG GATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGA TGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACC AAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTC GATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATG GCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTC ATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGC TACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCG TGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTT CTTGACGAGTTCTTCTGACTGTCAGACCAAGTTTACTCATATATACTTTAGAT TGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTG ATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCA GACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGC CGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCG CAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAA GAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGG CTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAG TTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCC CAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTAT GAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGC GGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTG GTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTT TGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCC TTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGC GTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATA CCGCTCGCCGCAGCCGAACGACCGA

SEQ ID NO.	Description	Sequence
68	linker	GGGG
69	Pep-O	VAPVGMHIANGM
70	AccA peptide 17	GSYHVMFMGLKKQLK

7. EXAMPLES

[00411] The following is a description of various methods and materials used in the studies. They are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention, nor are they intended to represent that the experiments below were performed and are all of the experiments that may be performed. It is to be understood that exemplary descriptions written in the present tense were not necessarily performed, but rather that the descriptions can be performed to generate the data and the like associated with the teachings of the present invention. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, percentages, etc.), but some experimental errors and deviations should be accounted for.

7.1 Example 1: *E. coli* strain engineering for OMV release and minimizing risk of reactogenicity

[00412] Escherichia coli (*E. coli*) strains are genetically engineered to optimize the release of outer membrane vesicles (OMVs) while concurrently reducing the risk of reactogenicity associated with their administration.

[00413] Homologous recombination techniques are utilized for *E. coli* engineering. The engineered *E. coli* strains exhibit a favorable phenotype characterized by enhanced OMV release and reduced reactogenic potential.

[00414] General molecular biology techniques are employed to manipulate and characterize the genetic modifications within the engineered *E. coli* strains.

[00415] The engineered *E. coli* strains are cultured under conditions conducive to OMV production. Subsequently, OMVs are harvested from the bacterial culture supernatant through centrifugation and filtration techniques. The isolated OMVs are assessed for their quantity, purity, and functional properties.

7.2 Example 2: *E. coli* strain engineering for heterologous glycan production in the OMVs

[00416] *E. coli* strains are genetically engineered to optimize to express the glycan antigens in outer membrane vesicles (OMVs).

[00417] Homologous recombination techniques are utilized for *E. coli* engineering. The engineered *E. coli* strains produce OMVs comprising the glycan antigens.

[00418] General molecular biology techniques are employed to manipulate and characterize the genetic modifications within the engineered *E. coli* strains.

[00419] Following genetic engineering, the engineered *E. coli* strains are cultured under controlled conditions conducive to OMV production. Subsequently, OMVs are isolated from the bacterial culture supernatant through centrifugation and filtration techniques. The presence of glycan antigens within the OMVs is confirmed through immunoblotting techniques.

7.3 Example 3: Construction of protein antigen expression plasmids and protein expression in the OMVs

[00420] *E. coli* strains are genetically engineered to introduce plasmids containing genes encoding the desired protein antigens.

[00421] Engineered *E. coli* cells harboring the protein antigen expression plasmids are cultured under controlled conditions to induce protein expression and OMV production. Immunoblots are conducted to confirm the presence of the expressed protein antigens within the OMV fraction.

7.4 Example 4: Production of OMVs in bioreactor (USP)

[00422] The engineered *E. coli* cells containing are cultured in a bioreactor system designed for optimal OMV production. The growth conditions within the bioreactor are controlled to provide an environment conducive to bacterial growth and OMV formation.

[00423] Subsequently, the cells are allowed to proliferate under controlled conditions, with continuous monitoring of growth parameters throughout the cultivation process. Upon reaching the desired cell density and growth phase, the culture broth containing OMVs is harvested from the bioreactor. The harvested OMV-containing supernatant is then processed further to isolate and purify OMVs.

7.5 Example 5: Purification procedure for OMVs (DSP), yield quantification via total protein

[00424] The culture broth containing OMVs harvested from the bioreactor is subjected to initial clarification steps to remove cellular debris and large particles. This may involve centrifugation or microfiltration to obtain a clarified supernatant enriched with OMVs.

[00425] The clarified supernatant then undergoes tangential flow filtration, a membrane-based separation technique. TFF is employed to concentrate the OMVs while simultaneously exchanging the buffer solution, thereby facilitating the removal of impurities and contaminants.

[00426] Following TFF, the concentrated OMVs are further purified using size-exclusion chromatography. This chromatographic technique separates biomolecules based on their size and molecular weight, allowing for the isolation of OMVs from smaller contaminants and protein aggregates.

[00427] This purified OMV fraction is subjected to subsequent analyses or applications. The yield of purified OMVs is quantified by measuring the total protein content in the purified OMV fraction.

7.6 Example 6: Assessment of antigen expression on OMVs

[00428] Immunoblots are performed to qualitatively assess the expression of specific protein and glycan antigens on the purified OMVs. ELISAs are employed as a more quantitative method to measure the expression levels of protein and glycan antigens on the OMVs.

7.7 Example 7: Analysis of OMV purity and morphology

[00429] Various techniques are employed to analyze the purity and morphology of the outer membrane vesicles (OMVs) obtained from the production process.

[00430] Size-exclusion chromatography coupled with high-performance liquid chromatography (SEC-HPLC) is utilized to assess the purity and size distribution of the OMV samples. SEC-HPLC analysis provides valuable information regarding the overall purity of the OMV preparations.

[00431] DNA quantification is performed to determine the concentration of DNA in the OMV samples.

[00432] Dynamic light scattering (DLS) and electron microscopy techniques are employed to characterize the size and morphology of the OMVs. DLS provides information on the diameter of the OMVs. Electron microscopy, including transmission electron microscopy (TEM) or scanning electron microscopy (SEM), allows for visualizing the morphology and size distribution of the OMVs.

[00433] The residual supernatants obtained after ultracentrifugation, which separates OMVs from other cellular debris and contaminants, are analyzed to ensure the purity of the OMV preparations. It is analyzed by Commassie staining to prove that no soluble protein contaminants are left in the preparation.

7.8 Example 8: Analysis of OMV composition

[00434] Total protein characterization techniques such as Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) and two-dimensional gel electrophoresis (2D gel) are utilized to identify and quantify the protein components present in the OMVs. Lipidomics analysis is utilized to determine the chemical composition in the lipids of the OMVs.

[00435] Total protein quantification is performed using the DS (dye-binding) assay for quantifying protein concentration in OMV samples. Additionally, amino acid quantification is carried out to provide an accurate measurement of protein content.

7.9 Example 9: Analysis of glycan antigen structure

[00436] The glycan antigen structure present on outer membrane vesicles (OMVs) is further analyzed using mass spectrometry. Prior to this analysis, the presence of glycan antigens on OMVs has been validated through Immunoblot and ELISA assays. Mass spectrometry assay is utilized to confirm the entire glycan structure, including the identification of specific glycan species that may not contain the antigens, if present.

7.10 Example 10: Lipid A modifications

[00437] Molecular biology techniques are employed to engineer *E. coli* strains for modifying Lipid A structures in OMVs. This includes genetic manipulation to alter the expression of enzymes involved in Lipid A biosynthesis pathways.

[00438] The functionality of modified Lipid A structures is assessed using an in-vitro assay involving reporter cell lines expressing Toll-like receptor 4 (TLR4). This assay evaluates the binding affinity of modified Lipid A variants to TLR4, and therefore resulting in different adjuvating effects. This assay is also used to select lead candidates with the highest but safest TLR4 activation profiles. Mass spectrometry techniques are further utilized to characterize the actual structure of the Lipid A variants in OMVs.

7.11 Example 11: Animal studies

[00439] Mice are immunized with OMVs. Various assays are performed using sera obtained from mice immunized with OMVs to evaluate their antibody response and functional activity against Neisseria gonorrhoeae.

[00440] Following immunization, serum samples are collected from the immunized mice, and the levels of immunoglobulin G (IgG) antibodies are quantified using enzyme-linked immunosorbent assay (ELISA). This quantitative assay measures the level of antigen-specific IgG antibodies present in the sera, providing a measure of the immunogenicity of the OMVs.

[00441] The functionality of the sera in mediating the killing of Neisseria gonorrhoeae by the complement system is evaluated using the serum bactericidal assay (SBA). Similar to SBA, the functionality of the sera in mediating bacterial killing is evaluated using the opsonophagocytic assay (OPA).

[00442] Specific assays are conducted to assess the sera functionality in antigen-specific assays. These assays include adhesion assays for NHBA and MetQ, analysis of Met(O) binding to MsrA/B blocking, and nitrite reductase assays for AniA.

7.12 Example 12: Pyrogenicity studies

[00443] Rabbits are immunized with the OMVs. Subsequently, the rabbits are evaluated for pyrogenicity. For example, blood samples may be collected to measure inflammatory markers or cytokine levels.

[00444] The MAT assay is also used to assess the pyrogenic potential of the OMVs. In this assay, monocytic cells are exposed to the OMVs, and the activation of these cells is measured by evaluating the release of cytokines.

7.13 Example 13A: *E. coli* strain engineered for the release of OMV expressing the target antigens, bearing modified lipid A, and engineered for minimizing the risk of reactogenicity

[00445] In order to construct target antigen-producing OMVs, a host organism (*E. coli* strain) was engineered with the following characteristics:

(a). inducible hypervesiculation;

(b). biosynthesis of the LOS (lipooligosaccharide) of *Neisseria gonorrhoeae* including the glycan antigens 2C7 epitope and KDO epitope and simultaneous inactivation of potentially interfering glycosyltransferases;

(c). balanced immune modulation by the modification of the lipid A structure and removal of flagellin and flagellin from the genome; and

(d). heterologous expression of the protein antigens AniA, MetQ, NHBA and MSrA/B.

[00446] Therefore, an *E. coli* K12 C600-derivative strain was constructed by subsequent replacements of the targeted gene clusters with the gene of interest, if any, followed by an FRT sites-flanked selection marker via λ-Red homologous recombination followed by FLP recombinase-catalyzed marker removal as described (TE Kuhlman and EC Cox, *Nucleic Acids Res*, 2010, Apr; 38(6): e92). The gene modifications of the engineered *E coli.* is listed in Table 1 with pLTPro231.

7.13.1 Inducible hypervesiculation

[00447] Three homologous recombination / marker removal steps were carried out, removing or replacing genomic sequences of Braun's lipoprotein *lpp* (GenBank NZ_CP031214 position 2,164,860 to 2,165,096), and of outer membrane protein A (*ompA*) (GenBank NZ_CP031214 position 2,901,443 to 2,902,483). The *lpp* gene was re-introduced in the attλ site under the promoter Para using the CRIM plasmid method described in Haldimann A and Wanner BL, *J Bacteriol*, 2001;183(21):6384-6393.

7.13.2 Biosynthesis of the LOS of Neisseria gonorrhoeae including the glycan antigens 2C7 epitope and KDO epitope and inactivation of potentially interfering glycosyltransferases

[00448] Three homologous recombination / marker removal steps were carried out, removing or replacing genomic sequences of

(i). clusters for the biosynthesis of *E. coli* K12 LPS core *waaQGPSBORYZU* (GenBank NZ_CP031214 position 117,878 to 127,774), which was replaced by an artificial operon containing genes for the biosynthesis of the LOS *lgtA* (*N. gonorrhoeae* 1291, GenBank ID AAF14358), *lgtB* (*N. mengitidis* MC58, GenBank ID AAF42257), *lgtE* (*N. gonorrhoeae* 1291, GenBank ID AAF14363), *lpt3* (*N. mengitidis* C311, GenBank ID QXZ29326), *lgtF* (*N. mengitidis* MC58, GenBank ID AAF42052), *rfaK* (*N. mengitidis* MC58, GenBank ID AAF42053), *lgtG* (*N. mengitidis* M22790, GenBank ID AOA42063), controlled by the existing genomic promoter;

(ii). the genomic locus *wza-wbbL* (GenBank NZ_CP031214 position 1,786,050 to 1,822,024), including the colanic acid cluster *wca*, *galF*, and the O antigen cluster *rfb*, which was replaced by *lst* from Neisseria gonorrhoeae 1291 (GenBank AAY41933) and further copies of *lgtB*, *lgtA* and *lgtE*, under the control of the constitutive promoter of *E. coli*'s *rfb* cluster which had been reinserted; and

(iii). enterobacterial common antigen (ECA) cluster (*wecA – rffM* (also known as *wecG*), GenBank NZ_CP031214 position 4,557,910 - 4,545,870), which was replaced by further copies of *lgtB* and *lgtA*.

[00449] An additional copy of the *lst* (SEQ ID NO: 37) from *Neisseria gonorrhoeae* (GenBank CP078119, positions 2,138,146 - 2,139,282) was introduced into a pBAD-derived plasmid under constitutive phage promoter (see Table 1, plasmid pLTPro159). 3-deoxy-manno-octulosonate cytidylyltransferase kdsB (SEQ ID NO: 53) from E. coli (GenBank NZ_CP031214 position 2,949,898 to 2,950,644) was introduced on a pEXT20 expression plasmid under an IPTG-inducible promoter via standard cloning techniques (see Table 1, plasmid pLTPro231, **FIG. 1A**). The plasmid construction is described in section 7.13.4. The genetic modification described in this

paragraph were the result of an iterative test of several variants in terms of integration locus, number of gene copies, gene origin, and gene orders.

[00450] The selection of the variants was based on the expression level of the glycan antigens via immunoblots (see Example 14), and the homogeneity of glycan species via silver staining (see Example 19).

7.13.3 Balanced immune modulation by the modification of the lipid A structure and removal of flagellin and fimbriae from the genome

[00451] A series of genetic modification were applied in order to achieve a lipid A structure similar to MPL® (Needham BD *et al.*, *Proc Natl Acad Sci U S A*, 2013;110(4):1464-1469; Wang YQ *et al.*, *Front Immunol*, 2020;11:577823; Hagen SR *et al.*, *J Chromatogr A*. 1997;767(1-2):53-61). Moreover, unwanted TLR activators flagellin and fimbriae were inactivated by deletion of the encoding genes.

[00452] Five homologous recombination / marker removal steps were carried out, removing or replacing genomic sequences of

(i). lipid A 1-diphosphate synthase *lpxT* (GenBank NZ_CP031214 position 1,653,666 - 1,652,952), which was replaced by the lipid A 1-phosphatse *lpxE* from *Francisella tularensis* subsp novicida U112 (GenBank CP009633.1 position 1715820 - 1715101) preceded by the constitutive lambda PL promoter;

(ii). the promoter of the lipid A palmitoyltransferase *pagP* (GenBank NZ_CP031214 position 3,264,939 - 3,264,378), which was replaced by the constitutive lambda PL promoter;

(iii). the gene coding for the phosphoethanolamine transferase EptA (*eptA*, GenBank NZ_CP031214 position 1,982,323 - 1,983,295), which was replaced by the gene for lipid A 3-O-desacylase PagL from *Salmonella enterica* (*pagL*, CP123648.1, position 1710552 – 1711114) preceded by the lambda PL constitutive promoter (REF);

(iv). flagellin (*fliC*, GenBank NZ_CP031214, position 1,786,050 - 1,808,792); and

(v). *fim* cluster, responsible for fimbriae biosynthesis (*fimHGFDCIA*, GenBank NZ_CP031214, position 3,975,452 – 9,983,143).

7.13.4 Heterologous expression of the protein antigens AniA, MetQ, NHBA and MsrA/B

[00453] For the individual protein antigens to be exported to the periplasm and anchored to the outer membrane ensuring their incorporation into OMVs, the presence of an N-terminal signal sequence followed by a cysteine-terminated “lipobox” motif, followed by an unstructured “tether” sequence is necessary. By recognizing these sequences, Gram negatives’ SEC and LOL export

machineries carry out the protein transfer to the periplasm, cleavage of the signal peptide, lipidation of the newly N-terminal cysteine, and transfer to the outer membrane. The selected antigen proteins are naturally outer membrane lipoprotein. In order to achieve the optimal outer membrane expression in *E. coli*, proteins were either kept with the original signal sequence and tether or their structured domains were fused with signal sequence and tether from another protein antigen. Expression plasmids were created via standard cloning techniques. Wild-type full length MetQ (SEQ ID NO: 5) and AniA (SEQ ID NO: 1) naturally contain a signal sequence for the export to the periplasm as well as a lipidation residue, therefore full length MetQ (SEQ ID NO: 5) and amino acids 1 to 347 of AniA (SEQ ID NO: 2) were used as expression constructs. Amino acids 244 – 426 of NHBA and amino acids 35 – 183 of MsrA/B were fused to various N-terminal sequences to ensure export to the periplasm and anchoring to the outer membrane by lipidation. pEXT20 expression plasmids harboring different variations in term of gene order, signal sequence and tether, and promoter of metQ, aniA, NHBA, msrA/B, and, in addition, kdsB (see section 7.13.2), were generated and tested for expression of each protein antigen and tested for protein expression via immunoblot (see Example 14). The finally-selected plasmid (**FIG. 1A**) contained the following sequences under an IPTG-inducible promoter, from 5' to 3': amino acids 35 – 183 of MsrA/B N-terminally fused to amino acids 1 – 42 of AniA (SEQ ID NO: 13), KdsB (SEQ ID NO: 53), full length MetQ (SEQ ID NO: 5), amino acids 1 to 347 of AniA (SEQ ID NO: 2), amino acids 244 – 426 of NHBA N-terminally fused to amino acids 1 – 42 of MetQ (SEQ ID NO: 20).

[00454] The OMV-producing strain was obtained by transforming the engineered strain with the plasmid for *lst* expression described in section 7.13.2 and the plasmid for protein antigen and *kdsB* expression described in section 7.13.4. A scheme summarizing the genetic modifications and their roles is presented in **FIG. 4**.

7.14 Example 13B: *E. coli* strain engineered for the release of OMV expressing the target antigens, bearing modified lipid A, and engineered for minimizing the risk of reactogenicity

[00455] In order to construct target antigen-producing OMVs, a host organism (*E. coli* strain) was engineered with the same characteristics as Example 13A, including:

(a). inducible hypervesiculation;

(b). biosynthesis of the LOS (lipooligosaccharide) of *Neisseria gonorrhoeae* including the glycan antigens 2C7 epitope and KDO epitope and simultaneous inactivation of potentially interfering glycosyltransferases;

(c). balanced immune modulation by the modification of the lipid A structure and removal

of flagellin and flagellin from the genome; and

(d). heterologous expression of the protein antigens AniA, MetQ, NHBA and MsrA/B.

[00456] Same genetic modifications made in Example 13A were introduced into host organism, except the plasmid for protein antigen and *kdsB* expression described in section 7.13.2 and 7.13.4, to construct an *E. coli* K12 C600-derivative strain. The gene modifications of the engineered *E coli.* is listed in Table 1 with pLTPro302 instead of pLTPro231.

[00457] 3-deoxy-manno-octulosonate cytidylyltransferase *kdsB* (SEQ ID NO: 53) from *E. coli* (GenBank NZ_CP031214 position 2,949,898 to 2,950,644) was introduced on a pEXT20 expression plasmid under a constitutive J23105 promoter via standard cloning techniques (see Table 1, plasmid pLTPro302, **FIG. 1B**).

[00458] The finally-selected plasmid (**FIG. 1B**) contained two transcription promoters: an IPTG-inducible tac promoter, and a constitutive J23105 promoter. The following sequences were under the tac promoter control, from 5' to 3': amino acids 35 – 183 of MsrA/B N-terminally fused to amino acids 1 – 42 of AniA (SEQ ID NO: 13), and amino acids 244 – 426 of NHBA N-terminally fused to amino acids 1 – 42 of MetQ (SEQ ID NO: 20). The following sequences were under the J23105 promoter control, from 5' to 3': full length MetQ (SEQ ID NO: 5), amino acids 1 to 347 of AniA (SEQ ID NO: 2), and KdsB (SEQ ID NO: 53).

7.15 Example 14: Production of OMVs in bioreactor, purification of OMVs, and preliminary analysis of recombinant antigens expression

[00459] The OMV-producing strain described in Examples 13B was grown in a batch 2-L bioreactor in a buffered rich medium- containing 50 g/L glycerol at 37°C or 35°C, and pH 7.1 mM IPTG was added after 4 hours. Cells were harvested by centrifugation 9 hours after inoculation and the supernatant containing OMVs was separated from the pelleted cells and subsequently filtered through 0.45 and 0.2 μm filters.

[00460] For a first purification step, the filtrated supernatant was adjusted to a final concentration of 74 mM Tris-HCl pH 8.15 then subsequently applied to tangential flow filtration (TFF) using a 300 kDa mPES Minikros 490 cm^2 1 mm hollow fiber filter module. The TFF permeate was discarded, the filter module was washed once and the OMV containing retentate was concentrated, diafiltrated with a two-buffer system (1×TBS, Tris Buffer Saline pH 8.5) and again concentrated. The OMVs were further purified by a size exclusion chromatography (SEC) step using a HiPRep 26/60 Sephacryl S-500 HR and another final TFF step following the same protocol as for the first step. The elution profiles were followed with absorbance at 280 nm. The purified OMVs were analyzed by SDS-PAGE gels followed by (i). coomassie staining, (ii). anti-

MetQ/AniA/MsrA/B/NHBA-blot, (iii). 2C7-immunoblot (using monoclonal antibody 2C7), (iv). anti-KDO immunoblot (using monoclonal antibody 6E4). The results of these experiments are shown in **FIGS. 5A-5E** and **FIGS 6A-6E** and are described in the following paragraphs. Further analytics, such as the ones described in the other experiment section, were also applied.

[00461] **SDS PAGE.** The amount of cells from overnight cultures corresponding to 1 mL of culture with OD600nm = 10 (measured using a spectrophotometer) was harvested by centrifugation. The OMV-containing supernatant was transferred into a fresh tube, supplemented with Lämmli-buffer and incubated at 95°C for 10 min prior to further analysis by SDS PAGE and Western blot. The cell pellets were resuspended in 100 µl of 4× Lämmli buffer and incubated at 95°C for 10 min prior to further analysis by SDS PAGE and Western blot. 10 µl of the sample were loaded on polyacrylamide gel and standard SDS-PAGE procedure followed by Coomassie staining. As shown in **FIG. 5A** and **FIG. 6B**, the engineered *E. coli* strain produced OMVs that could be successfully purified by the applied purification methods indicated by a reduced number of protein bands on SDS PAGE after each individual purification step as well as the co-elution and enrichment of a collection of proteins in a single well defined peak during size exclusion chromatography, which was indicative for intact OMVs.

[00462] **Immunoblotting.** Polyacrylamide gels after SDS PAGE were used for transfer of material on blotting membranes, which were then incubated with the proper primary antibodies and secondary HRP (horseradish peroxidase)-conjugated antibodies, which allowed color development through incubation with DMB (1,2-diamino-4,5-methylenedioxybenzene). In particular, monoclonal antibody 6E4 (Jen FE *et al., mBio*, 2021;12(2):e03666-20) was used for detection of the glycan KDO epitope, 2C7 monoclonal antibody (Yamasaki R *et al., J Biol Chem*, 1999;274(51):36550-36558. doi:10.1074/jbc.274.51.36550) was used for detection of 2C7 epitope counterpart, and a mix of four anti-protein antisera, obtained by immunizing animals with soluble versions of the protein antigens, was used for the protein blots. Results are reported in **FIGS. 5B-5D** and **FIGS. 6C-6E**. The immunoblot analysis confirmed the presence of the antigens in the produced OMVs, indicated by the proof of antigen identity during different steps of the OMV production.

[00463] The OMV-producing strain described in Example 13A was also produced, purified, and analyzed using the methods described in Example 14 and similar results were generated by using this strain (data not shown).

7.16 Example 15: Quantitative analysis of OMVs' total protein content

[00464] The yield of produced OMVs per cell mass was indirectly assessed by quantifying the total protein content after purification. The protein amount was determined by DC (detergent compatible) protein assay, which was based on colorimetric reactions of the peptide bond and aromatic residues under alkaline conditions. This assay was similar to the Lowry assay (Lowry OH *et al., J Biol Chem*, 1951;193(1):265-275), however the colorimetric reaction took place after detergent solubilization of the protein containing sample. Different dilutions of the analyte were mixed with SDS to a final concentration of 2% and incubated at 94°C for 10 minutes. The reagents from the DC Protein Assay kit (BioRad) were then mixed, and the absorbance at 750 nm was measured with a spectrophotometer after 15 minutes. The total protein amount was calculated based on a standard curve obtained with BSA. The value of protein amount determined by the DC protein assay had been cross-validated by amino acid analysis, which was considered the most accurate method for total protein quantification. During amino acid analysis, the absolute amounts of individual amino acids were quantified by hydrolysis of the proteins into amino acids and their subsequent quantification by ion exchange chromatography and post-column derivatization with ninhydrin. The samples were subjected to an acid hydrolysis performed for 20 hours at 110°C, in 6 M hydrochloric acid, 0.1% phenol, 0.1% thioglycolic acid under reduced pressure in an atmosphere of nitrogen. Identification and quantification of the amino acids was performed on a BioChrom 30 amino acid analyzer using ion exchange chromatography, post-column derivatization with ninhydrin and detection at two wavelengths, 570 nm and 440 nm. The total protein content was calculated as the sum of the individual 18 amino acids. The results obtained comparing the two methods are reported in Table 2.

Table 2: Quantitative analysis of OMVs by CD protein assay and amino acid analysis

sample	Protein amount by DC (ug/ml)	Protein amount by amino acid analysis (ug/ml)
Research batch A	1.28	1.16
Research batch B	2.83	2.27

[00465] The DC assay is a reliable method to quantify the protein amount in OMV containing samples since it is reproducible and could be validated by amino acid analysis. It was therefore used for dose calculation for any further assays. Based on this assay it was possible to calculate the OMV yield per volume of fermentation.

7.17 Example 16: Analysis of OMV purity and morphology via SE-HPLC (Size Exclusion High Pressure Liquid Chromatography), DLS (Dynamic Light Scattering), EM (Electron Microscopy), and qPCR (quantitative polymerase chain reaction)

[00466] OMVs were generated from the OMV-producing strain of Example 13A. SE-HPLC (Size Exclusion High Pressure Liquid Chromatography) was used to further characterize OMV containing samples during and after the purification process regarding their purity and homogeneity. 2.5 µg of total protein as determined by DC assay were subjected to a SE-HPLC using an HPLC device equipped with autosampler, UV-vis detector, and an Agilent Bio SEC-5 liquid chromatography column. The running buffer was TBS. The elution profiles were followed with absorbance at 215 nm. **FIG. 7A** shows elution profiles of an OMV- containing sample after the TFF-step, a sample after SEC and a sample after the second TFF-step. All three profiles showed two main peaks at a retention time of 8.3 and 11.5 min, however after each consecutive purification step the second peak at 11.5 min was reduced compared to the sample after TFF resulting in a final relative purity of the sample of 98%. As for the SEC purification step, SEC-HPLC confirmed that the purified OMVs eluted as one single species, confirming the high purity of the final samples.

[00467] DLS (Dynamic Light Scattering) measurements with a WYATT DynaPro NanoStar were carried out to assess the hydrodynamic radius of the purified OMVs. Samples of purified OMVs of a protein concentration of 10 ng/mL were used and a cumulative fit was applied for data fitting. DLS measurements show that purified OMVs were mainly monodisperse and with an average hydrodynamic radius of 50-60 nm.

[00468] Negative stain EM (Electron Microscopy) was used to monitor the morphology of the OMV and confirm the determined radius. 4 µl of OMV analyte (containing approximately 0.4 µg total protein) were adsorbed on Carbon Film 400 Mesh, Cu, 50/bxCF400-Cu-50 grids and dried by blotting with Whatman paper after 30 seconds. Two washes with water droplets followed by Whatman paper blotting and two staining cycles with uranyl acetate drops followed by Whatman paper blotting were carried out next. The grids were air-dried and observed with a TFS Talos L120C transmission electron microscope (Thermo Fisher) operating at 120 kV. Electron micrographs were recorded at a nominal magnification of 105,000X. EM results confirmed DLS findings as shown in **FIG. 7B**, where well dispersed round vesicles with a size distribution of 37 – 66 nm were observed, indicating that the engineered *E. coli* strain produced vesicles of uniform shape, which remained intact during all steps of the purification process.

[00469] qPCR (quantitative polymerase chain reaction) was used to determine the amount of host cell DNA from the engineered *E. coli* host strain in the purified OMV samples. This method calculates the initial DNA template concentration as a function of the PCR cycle number. A commercially available kit (ThermoFisher, resDNASEQ™ Quantitative *E. coli* DNA Kits, Catalog number: 4460366) was used for DNA quantification. Briefly, the PCR product was detected using a TaqMan™ probe, a fluorescently labeled oligonucleotide binding specifically to *E. coli* DNA. During the combined annealing/extension phase of PCR this probe emitted a fluorescence signal which is proportional to the amount of accumulated PCR product. A standard curve was generated by using *E. coli* K12 as external DNA standard available from the kit. Before qPCR measurement, OMV samples were lysed and the DNA was extracted using magnetic beads. For each sample, 1 ng of total protein amount was used. For all tested batches of purified OMVs, the amount of co-purified host cell DNA was below the detection limit of 1.5 pg DNA/µg protein demonstrating that the applied purification protocol resulted in an OMV preparation free from *E. coli* derived DNA.

7.18 Example 17: Protein antigen quantification via Sandwich ELISA (Enzyme-Linked Immunosorbent Assay)

[00470] To determine the amount of the individual protein antigens relative to the total protein amount (as determined by DC), a sandwich ELISA was performed. Here, 100 µl monoclonal antibodies against the individual protein antigens at 5 µg/ml concentration were used for coating MaxiSorp 96-well plates (Nunc, Thermo Fisher) over night at 4°C. Plates were blocked with 2.5% BSA in PBST 1h agitating at 600 rpm. OMV samples were diluted to 5 µg/mL in PBS pH 7.5 and incubated 1:1 with 25 mM SDS for 10 minutes at 95°C, agitating at 1500 rpm. The sample was further diluted 5 to 160-fold and different dilutions were added to the wells. Plates were incubated for 1 h at room temperature at 600 rpm agitation. As primary antibodies, 125 µl of polyclonal antibodies (rabbit) against the individual protein antigens in a dilution of 1:10.000 were used and plates were incubated 1 h at room temperature at 600 rpm agitation. As secondary antibodies for detection, 125 µl of HRP-coupled anti-rabbit antibodies were used in a dilution of 1:2.000 and incubated 1 h at room temperature at 600 rpm agitation. Development was carried out by adding 125 µl TMB (3,3',5,5' – tetramethylbenzidine), agitating at 600 rpm at room temperature, stopping the reaction by adding 125 µl 2M sulfuric acid, and recording the absorbance at 450 nm. Standard curves with soluble recombinantly produced protein antigens were prepared for concentration calculation.

[00471] The following concentrations reported in Table 3 were measured for different research batches derived from OMV-producing strains having different genetic backgrounds and on lead OMV-producing strain candidate. The main differences between research batches are:

- Research batch A: Produced from OMV-producing strain with *film* cluster not deleted, incorporating plasmid pLTPro231, and having lipid A in "C" configuration as described in Example 20.
- Research batch B: Produced from OMV-producing strain with *film* cluster not deleted, incorporating plasmid pLTPro231, and having lipid A in "A" configuration as described in Example 20
- Research batch C: Produced from OMV-producing strain of Example 13A
- Research batches D and E: Produced from OMV-producing strain of Example 13B. Research batch D was produced by growing the OMV-producing strain at 35°C, and Research batch E was produced by growing the OMV-producing strain at 32.5°C.

Table 3: Protein antigen content of total OMV protein amount (%) as determined by sandwich ELISA. CV: Coefficient of Variation

Sample	Target protein percentage on total OMV proteins (as measured by DC)					CV (%)			
	AniA	MetQ	NHBA	MsrAB	Total all 4	AniA	MetQ	NHBA	MsrAB
Research batch A	3.23	0.59	0.5	0.04	4.4	14.1	1.6	3.1	9.9
Research batch B	2.48	0.97	0.42	0.11	4.0	3	2.1	22.2	17.7
Research batch C	1.11	0.16	0.21	0.02	1.5	15.4	1.1	8.9	3.5
Research batch D	4.08	6.44	0.97	1.71	13.2	15.5	3.8	8.5	11.8
Research batch E	0.75	1.63	0.51	0.28	3.2	7.2	6.5	6.5	6.7

7.19 Example 18: KDO and heptose monosaccharide quantification for the determination of glycan and lipid A amounts

[00472] Monosaccharide analyses were performed to determine the amount of glycan antigens and lipid A in the OMV sample.

[00473] To determine the KDO monosaccharide absolute amount, OMV samples were diluted to 20 µg/mL total OMV proteins and hydrolyzed by mixing 1:1 with 4M acetic acid to release KDO monosaccharide. In this step, all terminal KDO monosaccharide were released. Therefore, 2 moles of KDO monosaccharide were expected per complete glycan structure, one of which is included in the "KDO" epitope, while the other is part of the oligosaccharide inner core. KDO was separated from OMV material by means of a PES (polyethersulfone) spin column. The KDO-containing eluate was mixed 1:1 with DMB (1,2-diamino-4,5-methylenedioxybenzene) labelling solution. DMB is a fluorescent label which was then used for detection. 5 µl of the obtained derivatized KDO species were then analyzed by reversed phase ultra performance liquid chromatography with fluorescence readout. C18 reverse phase columns were employed, and fluorescence was detected. Standard curves with KDO were used to determine the absolute KDO concentration in the sample.

[00474] To determine the heptose monosaccharide absolute amount, OMV samples were first hydrolyzed with 2M trifluoroacteic acid (TFA) for 4 hours at 121°C to obtain the free monosaccharide from OMV samples. Free monosaccharide species were analyzed by HPAEC-PAD (high performance anion exchange chromatography coupled with pulsed amperometric detection) and compared to a heptose standard curve for its quantification.

[00475] The terminal antigenic KDO was present in the glycan species, due to the positive LOS Western blot with 6E4 monoclonal antibody, and the negative results when Lst, the KDO transferase, was not present (not shown). In Example 19, silver stain and mass spectrometry (MS)

analysis were reported. LOS silver staining produced homogeneous bands, but their assignment to specific species was not possible. The terminal antigenic KDO was labile to the conditions employed to prepare and analyze samples via MS, but except for the lack of terminal KDO, no intermediate glycan species were detected, confirming the silver staining readout. Two heptose moles were present in a mole of glycan; therefore the amount of glycan (excluding the terminal antigenic KDO) could be calculated. One mole of measured KDO monosaccharide belonged to the glycan core, while another mole was the antigenic terminal KDO, therefore from its measurement it was possible to calculate the ratio of glycan which possessed a terminal KDO extension, as the inner KDO was always present and required for the lipid A export to the outer membrane. The amount of heptose allowed also to indirectly calculate the lipid A amount, as one mole of lipid A is present for every two moles of heptose. The results obtained for OMVs derived from the strain of Example 13A and OMVs derived from the strain of Example 13B are reported in Tables 4A and 4B respectively.

Table 4A: Calculated amount of LOS and lipid A of OMVs (derived from the strain of Example 13A)

Species	**Calculated amount per 50 µg total OMV proteins**
Complete LOS (KDO+, 2C7+)	13.43 µg (70.6 % of glycan species)
Lacks terminal KDO (KDO-, 2C7+)	5.6 µg (29.4 % of glycan species)
Lipid A	14.3 µg

Table 4B: Calculated amount of LOS and lipid A of OMVs (derived from the strain of Example 13B)

Species	**Calculated amount per 50 µg total OMV proteins**
Complete LOS (KDO+, 2C7+)	8.04 µg (37.4 % of glycan species)
Lacks terminal KDO (KDO-, 2C7+)	12.15 µg (62.6 % of glycan species)
Lipid A	13.4 µg

7.20 Example 19: Analyses of the whole LOS (lipooligosaccharide) structure

[00476] To analyze the molecular species of the whole lipooligosaccharide (LOS), a combination of techniques was employed. LOS immunoblots with well characterized anti-glycan specific monoclonal antibodies confirmed the presence of both the glycan antigens (see Example 14). LOS silver staining allowed to determine the homogeneity of the glycan species. Mass spectrometry on either whole LOS or isolated lipid A allowed to confirm the glycan species (with exception of the terminal KDO, see Example 18), and allowed to analyze the structure of the lipid A.

[00477] **LOS silver staining**. OMV preparations were mixed with Lämmli buffer and heated at 95°C for 10 minutes. After cooling, proteinase K was added, samples were incubated at 55°C for 1 h, and finally heated 10 minutes at 70°C. An SDS-PAGE was loaded with the sample and ran. Next, the gel was treated as follows: soaking in fixing solution (40% v/v ethanol, 5% v/v acetic acid) for 1 hour, soaking in oxidizing solution (0.7% w/v periodic acid in fixing solution) for 15 minutes, washing with ddH_2O for 15 minutes three times, staining with staining solution (0.33% v/v NH_3, 19 mM NaOH, 0.67% w/v $AgNO_3$) for 15 minutes, washing with ddH_2O for 10 minutes three times, developing with developing solution (25% w/v citric acid monohydrate, 0.0005% v/v formaldehyde solution) until bands appeared, stopping the development by ddH_2O washes. In **FIG. 8A**, a unique band for Research batch B of Example 17 was observed, while the control OMV with no glycan ("rough" *E. coli*'s LOS) ran at a lower molecular weight. In **FIG. 8B**, a unique band for the OMVs generated from the strain of Example 13B was observed, while no low molecular species were detected. The band was at the expected size. The expected running behavior of a "rough" *E. coli* LOS is also reported for reference.

[00478] **MALDI-MS.** Complete LOS molecules (comprising both oligosaccharide and lipid A) were isolated from OMV preparations: a monophasic Bligh-Dyer extraction using ammonium acetate, methanol, chloroform and water was performed followed by an ethanol precipitation step and a desalting step. The isolated LOS was either directly analyzed by MALDI-MS or was further treated to isolate lipid A. To isolate the lipid A moiety from the LOS, the LOS was hydrolyzed with 1% acetic acid and the released lipid A was separated using a two-phase Bligh-Dyer with methanol and chloroform. After washing the sample three times with a two-phase Bligh-Dyer with methanol and chloroform, it was dried by using a nitrogen stream and resuspended in chloroform:methanol (4:1, v/v). MALDI-MS analysis were carried out using a Bruker ultrafleXtreme MALDI-MS System. In **FIGS. 9A & 9B**, the results from the intact whole LOS measurements are reported. The measured masses corresponded to LOS species with variable lipid A structure and constant glycan structure, the latter being the expected complete glycan devoid of the terminal antigenic KDO. Analogous lipid A species distribution was retrieved when measuring the released lipid A only (**FIGS. 10A & 10B**).

[00479] The structural analysis of the LOS confirmed the presence of a homogeneous glycan, with the exception of the terminal antigenic KDO (see Example 18 for its quantification). The mixture of lipid A species was analogous to the lipid A species measured for MPL®, used in GSK's adjuvant AS04 (Wang YQ *et al.*, *Front Immunol*, 2020;11:577823; Hagen SR *et al.*, *J Chromatogr A*. 1997;767(1-2):53-61).

7.21 Example 20: Immune responses elicited by OMV vaccine candidates in mice

[00480] **Immunization.** To test the ability of the OMV vaccine to raise functional immune response, immunization studies were carried out. BALB/c mice were immunized with OMVs expressing the 2 glycan epitopes 2C7 and KDO, as well as the 4 protein antigens AniA, MetQ, NHBA, MsrA/B according to the schedule reported in **FIG. 11A**. The lead candidate (produced from OMV-producing strain with *film* cluster not deleted, incorporating plasmid pLTPro231) having lipid A structure A was compared to OMVs which differ for the lipid A structure (B, C, D).

[00481] The genetic modifications introduced into the OMV-generating strains to produce OMV candidates A, B, C and D, which had lipid A structures A, B, C, and D respectively, are as follows:

- Lipid A Structure A - Δ*msbB*::*msbB* Δ*eptA*::*pagL* Δ*lpxT*::*lpxE* ΔP_{pagP}::PL
- Lipid A Structure B - Δ*msbB* Δ*lpxT* Δ*eptA*::*pagL*
- Lipid A Structure C - *ΔmsbB*
- Lipid A Structure D - *ΔmsbB ΔpagP*

[00482] Chemical structures of lipid A species in each of lipid A structures A-D are shown in **FIG. 3B** (lipid A structure A) and **FIG. 27A** (lipid A structure B), **FIG. 27B** (lipid A structure C), **FIG. 27C** (lipid A structure D).

[00483] Each group consisted of 10 mice. Mice were immunized with a high dose (25 μg of total protein) and a low dose (5 μg of total protein) for each OMV variant. All groups were immunized with alum as adsorbent. Control indicates animals that were immunized with either buffer and alum only or OMVs containing none of the 6 antigens.

[00484] **Immunogenicity. FIG. 11B** shows specific IgG titres (arbitrary units, AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0) and in terminal bleed sera ("post-III", at day 42) individual mice sera against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B as measured by ECLIA (electrochemiluminescence immunoassay, Bolton JS *et al., Malar J*, 2020;19(1):159). For the assay, biotinylated soluble protein antigens were used for coating. The four protein antigens expressed in *E. coli* derived OMVs of the lead candidate "A" with the 2 glycan epitopes 2C7 and KDO, as well as the 4 protein antigens AniA, MetQ, NHBA, MsrA/B were immunogenic against AniA, MsrA/B, NHBA, MetQ from *Neisseria gonorrhoeae*. Significant titer increase pre/post-III could be shown for the high and the low dose (**FIG. 11B**), confirming the ability of the lead candidate to raise specific immune responses against the protein antigens.

[00485] **Serum Bactericidal Activity (SBA).** To understand whether the raised antibodies are able to trigger complement-mediated killing of *Neisseria gonorrhoeae*, a Luminescent Serum

Bactericidal Activity (L-SBA) assay was used. Here, serial dilutions of sera obtained during mice immunization as described above were added to plates' wells containing human complement and log phase cultures of *Neisseria gonorrhoeae* strain FA1090 and incubated for 3 hours. Cell viability was measured by mixing with BacTiter-Glo and luminescence reading on a microplate reader, and IC50 values (sera dilution needed for 50% killing) were calculated. Pools of 2-mice-sera were pooled and analyzed. Results are reported in **FIG. 12A**, which shows that the lead candidate A elicited bactericidal sera.

[00486] Adherence assay. Gonococcal adherence assays were performed to further assess the functionality of the elicited antibodies. Here, monolayers of human cervical epithelial cells (tCX) were covered with *Neisseria gonorrhoeae* strain 1291 pre-incubated with serial dilutions of heat-inactivated mouse sera (pooled sera from 10 animals). Following the incubation, cell monolayers were washed three times with HBSS to remove non-adherent bacteria, epithelial cells were lysed in 1% saponin (which does not affect bacterial viability) and well contents was plated onto GC agar plates. Results were calculated as the mean CFU from three replicates and presented as percentage of adherent bacteria relative to non-treated adhesion. Statistical analysis performed with ANOVA and two-tailed Student's t-test. **FIG. 12B** shows the percentage of adherent bacteria relative to non-treated adhesion for sera from mice immunized with the four vaccine candidates A, B, C, D. For the high dose of all vaccine candidates, the adherence of *Neisseria gonorrhoeae* strain 1291 to human cervical epithelial cells was reduced upon incubation compared to alum-only and no-antigens controls indicating the functionality of the elicited antibodies. The lead vaccine candidate (A) showed a reduction of adherence for both, the high and the low dose of administered vaccine. The induced antibodies from the lead vaccine candidate (A) demonstrated functionality regarding their potential to interfere with adherence of gonococcal bacteria to human cervical epithelial cells which is one of the first steps of disease onset.

[00487] MsrA/B **substrate binding inhibition assay.** A competition assay by Surface Plasmon Resonance (SPR) was performed to assess the ability of the raised antibodies to block MsrA/B by blocking the binding to its native substrate methylsulfoxide (Met(O)). Recombinant MsrA/B was immobilized onto flow cells of a COOH5 Biosensor chip using amine coupling EDC-NHS reactions. Sera and Met(O) were flowed upon the immobilized protein or upon a no-protein control cell. Mouse sera were used as the first injection, and Met(O) as the second injection, with PBS used as a negative control. The competition was run for 60s and different dilutions of sera and amounts of Met(O) were assessed. Binding of Met(O) to MsrA/B was analyzed in presence and absence of serum, and with pre- and post-immune serum. Data was collected using the Pioneer Software

package and analyzed using Qdat analysis software. The calculated blocking values are reported in **FIG. 13**. These results showed that the elicited antibodies against MsrA/B in post III sera from all four vaccine candidates A, B, C, D were able to block binding of methylsulfoxide to MsrA/B, while the controls showed no activity as expected. This was particularly interesting as blocking activity was also observed for OMVs generating relatively low IgG titres against MsrA/B, suggesting high quality of the generated antibodies.

7.22 Example 21: Rabbit tolerability studies

[00488] Immunization. New Zealand white rabbits were immunized with the lead candidate produced from the strain of Example 13B in order to assess its tolerability. Rabbits were immunized with a high dose (HD, 50 μg of total protein) and a low dose (LD, 5 μg of total protein) of the lead vaccine candidate (Lead). The groups consisted of 5 animals each, which were immunized with alum as adsorbent with an additional group being immunized with the high dose but without alum (No alum) to assess the effect of alum. As controls, alum-formulated commercially available OMVs with wildtype lipid A (Invivogen, *E. coli* OMVs InvivoFit™, tlrl-omv, 50 ug), the commercially available vaccine Bexsero® (1 dose), and wP (whole cell Pertussis, WHO standard, NIBSC, 1 dose) were used. The immunization schedule is reported in **FIG. 14 A**.

[00489] Tolerability. Readouts for the tolerability were temperature monitoring and observation of local reactions. The temperature was prior to immunization (on three days: day -5, -4, -1 and 0 pre- immunization (-2h to -4h)) to determine the baseline temperature for each rabbit and verifying that it is lying within normal limits (38–40 °C). After immunization, the temperature of the 45 rabbits was monitored after each immunization (day 0, 14, and 28) once per hour over a period of 6 h. **FIG. 14 B** shows the measurement of the maximal temperature of 45 rabbits included in the study after i.m vaccine injection. The temperature increase of animals treated with the lead vaccine candidate at the high dose was comparable to the licensed vaccine Bexsero® and wp after three intramuscular injections. The temperature increased in animals treated with the lead vaccine lead candidate without alum and with InvivoFit™ OMVs indicating that alum as an adsorbent is required to minimize the pyrogenicity potential of the OMV vaccine and the chosen modified lipid A structure leads to a safer reactogenicity profile. To assess local reactogenicity, all animals were monitored pre-dose and at approximately 4-hour post-dose on Day 0 and daily for 3 consecutive days after each dosing (*i.e.*, days 0 to 3, 14 to 17, and 28 to 31), and, in case of local signs, daily until recovery. Local signs that were monitored were erythema and eschar formation, oedema formation, induration, hematoma and scab. After the first injection, 20% of the animals that received an injection with Bexsero® developed mild erythema and animals that received an

injection with wP (whole cell Pertusis) showed development of mild oedema. No other local reactions were observed for all groups after the first injection. After the second injection, 20% of the animals that received an injection with commercially available OMVs (InvivoFit™) demonstrated mild oedema and moderate induration. No other local reactions were observed for all groups after the second injection. After the third injection, 20% of the animals injected with the lead vaccine candidate and with commercially available OMVs (InvivoFit™) developed mild oedema and mild induration, no other local reactions were observed. The vaccine lead candidate was therefore considered to have a safe reactogenicity profile which is comparable to other licensed vaccine.

[00490] **Immunogenicity**. **FIG. 20** shows specific IgG titres (arbitrary units, AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0), in sera withdrawn after the second immunization ("post-II", at day 28), and in terminal bleed sera ("post-III", at day 42). Individual rabbit sera against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B were measured by ECLIA (electrochemiluminescence immunoassay, Bolton JS *et al., Malar J*, 2020;19(1):159). For the assay, biotinylated soluble protein antigens were used. The four protein antigens (AniA, MetQ, NHBA, and MsrA/B) expressed in *E. coli* derived OMVs of the lead candidate with the 2 glycan epitopes 2C7 and KDO were immunogenic. Significant titer increase pre/post-II and pre/post-III could be shown for the high and the low dose independently to the co-formulation with alum (**FIG. 20**), confirming the ability of the lead candidate to raise specific immune responses against the protein antigens.

7.23 Example 22: Monocyte activation test (MAT)

[00491] To assess the pyrogenicity and the immune activating potential of the OMV preparation in the vaccine candidate, a monocyte activation test (MAT) was performed. This assay measured the release of interleukin-6 (IL-6) by human peripheral blood mononuclear cells (PBMCs) upon contact with endotoxic substances. These substances are recognized by the cells via various Toll-like Receptors (TLRs) which induce the expression of interleukins. The amount of released IL-6 was detected by ELISA. Briefly, after incubation of human PBMCs with OMV samples in serial dilutions in a microplate, the samples were transferred to an anti-IL-6 microplate for ELISA measurements. Various OMV vaccine candidates (produced from OMV-producing strain with *film* cluster not deleted, incorporating plasmid pLTPro231) with different genetic backgrounds regarding lipid A structure (samples A, B, C, D, as described in Example 20 as lipid A structures A-D) were compared as well as commercially available OMVs with wildtype lipid A (Invivogen, *E. coli* OMVs InvivoFit™, tlrl-omv) and as internal control, an endotoxin standard was used. For this test,

the OMVs were not formulated in alum. **FIG. 15** shows the IL-6 concentrations measured after incubation of the individual OMV preparation with PBMCs. As can be seen, the genetic background regarding lipid A structure influenced IL-6 release and it is therefore possible to modulate the immune activating potential of the vaccine candidate by choosing the expression of a beneficial lipid A variant. The lead vaccine candidate (sample A) exhibited a lower immune activation potential than OMVs with wildtype lipid A but a higher immune activation potential than OMVs expressing lipid A variants (B, C, D). As the lead candidate A also showed a safe profile in rabbits tolerability studies, it was selected for its higher potential in stimulating a strong and safe immune response.

7.24 Example 23: Design and testing of additional cNHBA-MetQ fusion proteins

[00492] This example investigated different fragments and fusions of cNHBA and MetQ, and evaluated the vaccine potential of these antigens, *via* functional activity of antibodies raised against these fragments and fusions, to identify minimal and optimal antigens for gonococcal vaccine development. The cNHBA and MetQ fragments and fusion antigens were all immunogenic in mice, generating a predominantly IgG1 response. Antibodies mediated bacterial killing *via* both serum bactericidal activity (SBA) and opsonophagocytic activity (OPA), and reduced adherence to cervical and urethral epithelial cells. Among the antigen fusions tested, MetQ-cNHBA and cNHBA-Met2 generated the highest SBA, OPA and adherence blocking titres and are proposed as promising optimised antigens for *N. gonorrhoeae* vaccine development.

[00493] **Materials and Methods.**

[00494] *Cloning, expression and purification of cNHBA and MetQ fragments and fusion proteins.* cNHBA (C-terminal 183 amino acid fragment of NHBA, SEQ ID NO: 16), MetQ (SEQ ID NO 7), Met1 (MetQ N-terminal 105 amino acid fragment, SEQ ID NO: 57) and Met2 (MetQ C-terminal 166 amino acid fragment, SEQ ID NO: 58) were amplified from *N. gonorrhoeae* strain 1291 and cloned into pET19b (Novagen) with an N-terminal His tag. The fusion genes (MetQ-cNHBA (SEQ ID NO: 62), cNHBA-MetQ (SEQ ID NO: 65), Met1-cNHBA (SEQ ID NO: 60), cNHBA-Met1 (SEQ ID NO: 63), Met2-cNHBA (SEQ ID NO: 61) and cNHBA-Met2 (SEQ ID NO: 64)) were synthesized by Integrated DNA Technologies (IDT) with N-terminal His tags and a non-polar Gly-Gly-Gly-Gly linker (SEQ ID NO: 68) between the fused proteins. The N-terminal signal peptide sequence was not included in the cloned genes. The fusion genes were subcloned into pET-19b (Novagen) and protein expression and purification were performed. Briefly, *E. coli* BL21 (DE3) harbouring expression plasmids were grown to an optical density at 600 nm of 0.5 supplemented with 1% glucose. The temperature was then lowered to 20°C and grown an hour, and

expression was induced with 1mM IPTG for further 16 hours. After cell lysis *via* sonication, soluble and insoluble fractions were separated by centrifugation and recombinant proteins were purified using TALON His-tag purification resin (Takara Bio). Fractions were collected and concentrated through centrifugal filtration units (Amicon Ultra), centrifuged, and filtered through a 0.22 μm filtration unit (Millipore). Recombinant proteins were buffer exchanged into 50 mM HEPES, 100 mM NaCl, pH 8, incubated with 2 mM $CaCl_2$ and Enterokinase (NEB) at 25°C for 16 hours to cleave the His-tag. Benzamidine Sepharose (Cytiva) was added to the mixture and incubated for 15 minutes. Size exclusion chromatography was performed on Superdex 200 Increase 10/300 GL column (Cytiva) fitted to an ÄKTA purification system (Cytiva). Purity of the proteins was confirmed by SDS-PAGE and Western Blot analysis using anti-His (Bio-Rad), NHBA, or MetQ specific antibodies.

[00495] *AlphaFold2 and Molecular Dynamics (MD) structure prediction of protein antigens.* The 3D structures of all protein antigens (fragments and fusions) were generated using AlphaFold 2.0 and submitted to a Molecular Dynamics (MD) simulation using the AMBER96 forcefield implemented into the YASARA software suite (version 22.6.27.W.64). The cell size was set to 100 Å in each direction with periodic boundary conditions under physiological conditions (pH 7.4 with 0.9% NaCl atoms) at 298 K. A total of 50 ns capturing 501 snapshots were sampled. The structure with lowest energy was obtained from the trajectories excluding the first 25 ns as equilibration time. Alignment of simulated energy minimum structures with *N. meningitidis* NHBA (6CUJ) and MetQ (3GXA) x-ray crystal structures were performed with MUSTANG executed in YASARA.

[00496] *Generation of polyclonal antibodies.* Groups of five 8-week-old female BALB/c mice (Animal Resources Centre, WA, Australia) were immunized subcutaneously with 25 μg of recombinant protein with aluminium hydroxide (Alhydrogel; InvivoGen, San Diego, USA) on days 0, 21 and 28. Terminal bleeds were collected on day 42 and serum collected *via* centrifugation at 600 *g* for 10 minutes at room temperature. Pre-immune serum was also collected from each mouse prior to immunization.

[00497] *Enzyme-linked immunosorbent assays (ELISAs).* ELISAs were performed in triplicate using 96-well MaxiSorp (NUNC) plates coated with: 100 ng of purified NHBA or MetQ in 100 μL of coating buffer (0.5 M carbonate buffer, pH 9.6) at 4°C for 16 hours; or whole cells of *N. gonorrhoeae* strains 1291 or WHO X at 25°C for 16 hours. Plates were incubated with pooled mouse sera, and secondary antibody as specified in the results (horseradish peroxidase conjugated polyclonal rabbit anti-mouse IgG (Merck, Darmstadt, Germany) or goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Thermofisher Scientific, Waltham, USA)). The ELISA titer was reported as the

highest serum dilution with absorbance at 450 nm greater than the mean negative control (all reagents excluding primary antibody) plus 3 standard deviations.

[00498] *Serum bactericidal activity (SBA) and opsonophagocytic killing activity (OPA) assays*. Approximately $1x10^3$ colony forming units (CFU) of *N. gonorrhoeae* were incubated in serial dilutions of heat-inactivated (56°C for 60 minutes) pooled mouse sera for 15 minutes at 37°C. The SBA assay was initiated by adding the complement source (normal human serum pre-absorbed against *N. gonorrhoeae*), followed by incubation at 37°C, 5% CO_2 for 1 hour. The complement at the concentration used is not bactericidal on its own to wild-type *N. gonorrhoeae*. The OPA assay was initiated by adding the complement source and ~$1x10^5$ polymorphonuclear leukocytes (PMNs), followed by incubation at 37°C, 5% CO_2 for 60 minutes. Serial dilutions of the contents of triplicate wells were plated on GC agar and grown overnight at 37°C, 5% CO_2. The SBA or OPA titer was the highest antibody dilution which induced more than 50% killing in the assay. Statistical analysis was performed using one-way analysis of variance (ANOVA) and two-tailed Student's t-test. Experiments were repeated three times. Human sera and PMNs were collected from healthy donors with written informed consent.

[00499] *Epithelial cell adherence assays*. Monolayers of E6/E7 transformed primary human cervical (tCX) and urethral epithelial (tUEC) cells (provided by M.A. Apicella, University of Iowa, USA) were incubated at 37°C, 5% CO_2 for 10 minutes with ~$0.5x10^5$ CFU *N. gonorrhoeae* strain 1291 that had been pre-incubated with heat inactivated mouse sera for 30 minutes. Following infection, cells were washed three times with HBSS to remove non-adherent bacteria, cells lysed with GC broth supplemented with 1% saponin and well contents then plated onto GC agar. Results were calculated as the mean CFU from three replicate wells and presented as percentage of adherent bacteria relative to no antibody control. The adherence blocking titer was the highest antibody dilution which resulted in a >50% reduction in adherence. Statistical analysis was performed using one-way analysis of variance (ANOVA) and two-tailed Student's t-test.

[00500] **Results.**

[00501] *Design and in silico characterisation of cNHBA and MetQ fragments and fusions as optimised vaccine antigens.* To optimise NHBA and MetQ as vaccine candidates, fragments and fusions of these antigens were generated including the NHBA C-terminal (cNHBA), full length MetQ, MetQ N-terminal (Met1) and MetQ C-terminal (Met2), as well as MetQ, Met1 and Met2 fused to either the N or C-termini of cNHBA *via* a poly-glycine linker (**FIGS. 16A-16C**). Analysis of ~19,000 isolates in the PubMLST database indicates a high level of conservation in NHBA

(≥92.9% for full the length protein; ≥ 91.1% for N-terminal of NHBA; ≥ 96% for cNHBA) and MetQ (≥99.6-% for full length protein; ≥ 99.7 for Met1; ≥ 99.4 for Met2).

[00502] To investigate antigen structure and dynamics, 3D structures of the proteins and fusions were generated using AlphaFold2, subjected to 50 ns MD simulations, and compared to the available *N. meningitidis* x-ray crystal structures of the NHBA C-terminal (PDB 6CUJ; 96% identity to *N. gonorrhoeae* 1291 cNHBA) and MetQ (PDB 3GXA; 97% identity to *N. gonorrhoeae* 1291 MetQ) (**FIGS. 17A and 17B**). Structural alignment of the modelled gonococcal cNHBA with 6CUJ (RMSD of 1.04 Å) and MetQ with 3GXA (RMSD of 1.76 Å) by MUSTANG indicate that both proteins have highly similar structural architecture compared to their meningococcal homologues. The Met1 and Met2 fragments both retained their structural features and overall fold with close structural alignment to the meningococcal MetQ crystal structure (RMSD of 2.19 Å and 1.48 Å for Met1 and Met2, respectively). However, Met1 was more disordered than MetQ or Met2 (RMSF value of 5.27, 3.09, and 2.64 Å, respectively). Fusion of the antigens had little to no impact on the structure of the individual components, with high structural similarity to the original proteins (RMSD ≤2.11 Å for 3GXA and 6CUJ). However, the order of the antigens in the fusion affected the overall protein architecture, their compactness and plasticity. For example, the architecture of the cNHBA-MetQ and cNHBA-Met2 fusions was more rigid and compact than that of the MetQ-cNHBA and Met2-cNHBA fusions, in which the individual components were more segregated (RMSF 3.16 and 3.88 *vs* 7.39 and 8.58 Å; radii of gyration 25.75 and 25.93 *vs* 31.35 and 30.68 Å, respectively). The models suggest that this is caused by the long (28 and 39 amino acids, respectively) flexible hinge domains that link these nonrigid fusion proteins.

[00503] *Immunogenicity of recombinant cNHBA and MetQ fragments and fusions.* Sera from mice immunized with recombinant cNHBA and MetQ, and fragments and fusions of these proteins adjuvanted with aluminium hydroxide were assessed *via* ELISA against either whole cell bacteria or recombinant proteins. For the whole cell total IgG ELISA, sera generated against all antigens could detect native NHBA and MetQ on the surface of the homologous *N. gonorrhoeae* strain 1291 (*i.e.,* strain from which the antigens were derived) as well as the heterologous strain WHO X (**FIG. 19**).

[00504] For all antigens tested, IgG1 was the dominant isotype against recombinant NHBA and MetQ (**FIG. 19**). The ratio of IgG isotypes was typically IgG1>IgG2b>IgG3≥IgG2a, except for MetQ-cNHBA where IgG1>IgG2a>IgG2b>IgG3 for MetQ. The fragment and fusion proteins had at least equivalent, and usually 2-4 fold higher IgG1 titres relative to the cNHBA or MetQ antigens when used alone. For example, the MetQ-cNHBA fusion had 2-fold higher titres to both cNHBA

and MetQ, compared to the single antigens. However, when cNHBA and MetQ were mixed and immunised together, there was an equivalent titer against MetQ, but 4-fold lower titer to NHBA. IgA titres were equivalent between antigens for MetQ and varied 2-fold for NHBA. IgM titres varied 2- and 4-fold between antigens for NHBA and MetQ, respectively.

[00505] *Functional activity of antibodies raised to recombinant cNHBA and MetQ fragments and fusions.* The functional activity of antibodies raised to each of the antigens were tested in serum bactericidal activity (SBA) assays, opsonophagocytic killing (OPA) assays, and adherence blocking. All antigens elicited antibodies with functional activity and titres (reciprocal of the lowest serum dilution that gave >50% killing or reduction in adherence) above the unimmunised and alum only immunised mice in at least one assay (**Table 5**). For SBA and OPA assays, titres of 400-3,200 were seen for *N. gonorrhoeae* strains 1291 and WHO X, with the cNHBA-Met2 and MetQ-cNHBA fusions having the highest titres. The full length MetQ induced higher titres than the Met1 and Met2 fragments, and in most cases the protein fusions induced a more potent functional immune response than the individual antigens. However, up to a 16-fold difference in titres were seen depending on the order of proteins in the fusion. For example, cNHBA-Met2 > Met2-cNHBA and MetQ-cNHBA > cNHBA-MetQ with respect to both SBA and OPA titres. The SBA and OPA activity of the top two candidates was evaluated in a panel of five WHO reference strains (**Table 6**). cNHBA-Met2 induced a higher or equivalent SBA and OPA titer than MetQ-cNHBA for all strains, except for WHO K and WHO Z OPA titres.

Table 5. Serum bactericidal and opsonophagocytic activity of mouse sera raised against MetQ and NHBA fragments and fusion proteins

Vaccine candidate	SBA titre		OPA titre	
	1291	WHO X	1291	WHO X
unimmunised	200	200	200	200
Alum	200	200	200	400
Met1	200	200	200	200
Met2	400	200	800	200
MetQ	800	800	800	800
cNHBA	800	800	800	800
Met1-cNHBA	800	1,600	1,600	1,600
cNHBA-Met1	800	400	800	400
Met2-cNHBA	200	400	200	400
cNHBA-Met2	1,600	1,600	3,200	3,200
MetQ-cNHBA	1,600	1,600	3,200	3,200
cNHBA-MetQ	800	1,600	800	800
cNHBA + MetQ	400	800	800	800

SBA, serum bactericidal activity titre; OPA, opsonophagocytic titre. Titre = reciprocal of the lowest antibody dilution which induced >50% killing for SBA and OPA.

Table 6. SBA and OPA titres of mouse sera raised against the top 2 MetQ/NHBA fusion proteins against a WHO panel of *N. gonorrhoeae* strains

Vaccine candidate	SBA titre					OPA titre				
	WHO K	WHO P	WHO G	WHO X	WHO Z	WHO K	WHO G	WHO P	WHO X	WHO Z
Alum	<100	200	800	200	200	<100	100	1,600	400	<100
cNHBA-Met2	400	1,600	6,400	1,600	1,600	<100	800	6,400	3,200	400
MetQ-cNHBA	200	400	3,200	1,600	1,600	400	400	6,400	3,200	800

SBA, serum bactericidal activity titre; OPA, opsonophagocytic titre; (titre = reciprocal of the lowest antibody dilution which induced >50% killing for SBA and OPA) Values above the alum only immunised controls are shaded gray with higher fold change above alum for each strain shader darker gray.

[00506] For *N. gonorrhoeae* strain 1291 adherence to cervical epithelial and male urethral cells, 17-82% blocking of adherence was seen in the presence of antibodies (**FIGS. 18A and 18B**). The highest blocking of adherence was seen for antibodies to cNHBA-Met2 and MetQ-cNHBA for urethral and cervical epithelial cells, respectively.

[00507] **Discussion.**

[00508] Six protein-protein fusions that contained cNHBA fused *via* a poly-glycine linker to either the N or C-terminus of the full length MetQ, the MetQ N-terminal (Met1) or the MetQ C-terminal (Met2) (*i.e.,* MetQ-cNHBA, cNHBA-MetQ, Met1-cNHBA, cNHBA-Met1, Met2-cNHBA and cNHBA-Met2) were evaluated. These fusions elicited different antibody levels, isotype profiles and functional responses, possibly due to protein structure, stability, and/or exposure of epitopes. The cNHBA-Met2 and MetQ-cNHBA fusions had the highest titres in SBA and OPA assays against *N. gonorrhoeae* strain 1291 and were among the antigens with the highest IgG3 and IgG2b titres, particularly to NHBA. Immunoglobulin isotypes differ in their ability to activate complement and mediate bactericidal and opsonophagocytic activity. For example, antibodies targeting the *N. meningitidis* PorA antigen have a hierarchy of IgG3>>IgG2b>IgG2a>>IgG1 for serum bactericidal activity and IgG3>IgG2b=IgG2a>>IgG1 opsonophagocytic activity. The potent functional activity of IgG3 is attributed to its unique structural and physiochemical characteristics and its superior capacity to engage complement.

[00509] *N. gonorrhoeae* infection is a complex, multifactorial event where the bacteria avoid and actively suppress host immune responses to effectively negate a protective humoral immune response. Consequently, *N. gonorrhoeae* infection does not lead to enduring protection against reinfection, and the type of vaccine-induced immune response needed to protect against *N. gonorrhoeae* is unknown. Antibody-dependent complement and cell mediated killing, as well as blocking of key bacterial functions, have been shown to be important mechanisms of protection against bacterial infections. For example, SBA is a correlate of protection for the closely related bacteria *N. meningitidis*, OPA is involved in protection for *Streptococcus pneumoniae*, and antibodies with functional blocking activity are required to neutralise tetanus toxin. Therefore, the ability of cNHBA and MetQ fusions to induce antibodies with multiple mechanisms of actions was investigated, including bactericidal, opsonophagocytic, and function blocking activity (*i.e.,* ability to block gonococcal adherence to genital tract epithelial cells and prevent initial stages of colonisation). Antibodies induced by cNHBA-Met2 and MetQ-cNHBA elicited killing of multiple WHO *N. gonorrhoeae* references strains in SBA and OPA assays and reduce adherence of *N. gonorrhoeae* strain 1291 to cervical and/or urethral epithelial cells.

[00510] The use of a fusion protein as a vaccine antigen has the potential benefit of inducing an immune response that is more potent than that induced by the individual antigens, while also reducing the number of vaccine components to facilitate more efficient and cost-effective, large-scale vaccine manufacture. Understanding antigen structure can provide insights into the potential effects of fusing two antigens and aid the vaccine design. *In silico* simulations of the structures of

the cNHBA and MetQ fragments and fusions, based on the x-ray crystal structures available for the *N. meningitidis* NHBA and MetQ homologues, indicated that they have highly similar structural overall fold compared to their meningococcal homologues. The N-terminal of NHBA is predicted to be unstructured, while cNHBA has a 2-stranded β-hairpin and 8-stranded anti-parallel β-barrel. MetQ has two globular domains that are connected by a hinge region, with the methionine-binding site formed at the interface between the domains. The Met1 fragment includes 105 amino acids of domain 1 and Met2 consists of part of domain 1, including N239 that corresponds to N238 in *N. meningitidis* MetQ that is essential for high affinity Met binding, and all of domain 2. The modelling indicated the overall structure of the individual antigens is conserved in the fusions. However, the order of the proteins in the fusions affected their structural stability, flexibility, spatial positioning and overall compactness. For example, individual domains of Met2-cNHBA and MetQ-cNHBA were more separated from each other than cNHBA-Met2 and cNHBA-MetQ fusion antigens, which were predicted to form tighter and more rigid structures due to a shorter, more stable hinge region between the proteins.

[00511] The findings described herein supported the presently disclosed fusion proteins as attractive, optimised antigens for inclusion in a multicomponent vaccine. These fusion antigens induced a more potent immune response than the single antigens when used alone or in combination. The use of a fusion protein also could simplify antigen production, purification, and formulation by reducing the number of components in the vaccine.

7.25 Example 24: Immune responses elicited by lead candidates in mice

[00512] **Immunization.** To test the ability of the OMV vaccine to raise functional immune response, immunization studies were carried out. BALB/c mice were immunized with OMVs expressing the 2 glycan epitopes 2C7 and KDO, as well as the 4 protein antigens AniA, MetQ, NHBA, MsrA/B according to the schedule reported in **FIG. 21A**. Two batches of the lead candidate ("Lead 1" and "Lead 2") formulated with alum were compared to a batch devoid of alum ("Lead 2 no alum"), OMV devoid of gonococcal antigens ("OMV no Ag"), and to OMV carrying only the glycan ("OMV-glyc", containing the 2C7 and KDO epitopes). Each group consisted of 10 mice. Mice were immunized with a high dose (25 μg of total protein) for each OMV variant. Control ("Ctrl") indicates animals that were immunized with buffer and alum only. Lead 1 had the same structure as the Research batch B of Example 17, produced from OMV-producing strain with *film* cluster not deleted, incorporating plasmid pLTPro231, and having lipid A in "A" configuration as described in Example 20. Lead 2 was produced from OMV-producing strain of Example 13B.

[00513] **Immunogenicity. FIG. 21B** shows specific IgG titres (arbitrary units, AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0), in sera withdrawn after the second immunization ("post-II", at day 28), and in terminal bleed sera ("post-III", at day 42). Individual mice sera (for "pre" and "post-III") or pooled sera ("post-II") against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B were measured by ECLIA (electrochemiluminescence immunoassay, Bolton JS *et al., Malar J*, 2020;19(1):159). For the assay, biotinylated soluble protein antigens were used. The four protein antigens expressed in *E. coli* derived OMVs of the lead candidate "A" (also called Lead 2 in Example 24) with the 2 glycan epitopes 2C7 and KDO, as well as the 4 protein antigens AniA, MetQ, NHBA, MsrA/B were immunogenic against AniA, MsrA/B, NHBA, MetQ from *Neisseria gonorrhoeae*. Significant titre increase pre/post-II and pre/post-III was shown for the two lead candidate batches and for the batch devoid of alum (**FIG. 21B**), confirming the ability of the lead candidate to raise specific immune responses against the protein antigens. The same sera were analyzed via ELISA (Enzyme-Linked Immunosorbent Assay), where plates were coated with LOS containing the 2C7 epitope or the KDO epitope, or the LOS containing both the 2C7 and the KDO epitopes, all recombinantly produced in *E. coli*. Significant titre increase pre/post-III could be shown for the two lead candidate batches, for the batch devoid of alum, and for the batch where only glycan antigens were present (**FIG. 22**).

[00514] **Serum Bactericidal Activity (SBA).** To understand whether the raised antibodies were able to trigger complement-mediated killing of *Neisseria gonorrhoeae*, a Luminescent Serum Bactericidal Activity (L-SBA) assay was used. Here, serial dilutions of sera obtained during mice immunization as described above were added to plates' wells containing human complement and log phase cultures of *Neisseria gonorrhoeae* strain FA1090 and of strain WHO-R and incubated for 3 hours. Cell viability was measured by mixing with BacTiter-Glo and luminescence reading on a microplate reader, and IC50 values (sera dilution needed for 50% killing) were calculated. Individual mice-sera after 3 immunizations (post-III) were analyzed. **FIG. 23** shows that the lead candidate batches elicited bactericidal sera.

7.26 Example 25: Immune responses elicited by single-antigen OMV in mice

[00515] **Immunization.** To test the ability of the selected gonococcal antigen to elicit an immune response when expressed on OMV, immunization studies were carried out. BALB/c mice were immunized with 25 μg (in total protein) of OMV batches expressing i. AniA, ii. NHBA, iii. MsrA/B, iv. MetQ, v. KDO, or vi. KDO and 2C7, compared to 25 μg (in total protein) of OMV without antigens, 5 μg of each of the four protein antigens (soluble versions), and to a buffer

control. All groups were immunized with alum as adsorbent. Each group consisted of 10 mice. The immunization schedule is reported in **FIG. 24**.

[00516] Immunogenicity. FIG. 25 shows endpoint titres in pre-immunization sera ("pre", or "Pre-bleed", at day 0) and in terminal bleed sera ("post-III", at day 42) of pooled mice sera against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B as measured by ELISA (Enzyme-Linked Immunosorbent Assay), where plates were coated soluble versions of the four protein antigens. Protein antigens expressed on OMV were immunogenic (**FIG. 25**). "Control" indicated the relative soluble protein used for immunization.

7.27 Example 26: Binding of live *N. gonorrhoeae* by raised sera in rabbit

[00517] Rabbit immune sera obtained by immunizing with two different doses of lead candidate OMV ("Lead", 50 µg total OMV proteins and "Lead-MD" (lead with medium dose), 7.3 µg total OMV proteins, expressing 2C7, KDO, AniA, MetQ, NHBA, and MsrA/B; similarly to example 21, the lead OMVs were produced from the strain of Example 13B), OMV without antigens ("OMV no Ag"), or by Bexero® were analyzed for their ability to bind different strains of live *N. gonorrhoeae* cells. Strains "WHO F", "WHO Z", "WHO R", "WHO K", "1291", "1263546", "1087092", "WHO S2", and "FA1090" were grown overnight on Chocolate PolyVitex agar. Liquid media were inoculated from plates until reaching an optical density at 600nm of 0.5. Cultures were then added to 96-well plates, washed, and incubated with 1:100 serum (or with rabbit monoclonal 2C7 antibody – Rb2C7 - as positive control or with buffer only as negative control). Anti-rabbit IgG secondary antibody coupled with Alexa 488 dye was then added for detection after a washing step. Bacteria were then inactivated with 1% paraformaldehyde and plate acquisition was carried out by FACS Cytoflex LX (Beckman) using the 525/40 BP channel (B525) for Alexa 488. Data were analyzed using FlowJo. Results are reported in **FIG. 26**. Sera raised by the lead candidate were able to bind all the tested strains. WHO strains were provided by WHO; 1263546 and 1087092 were provided by JMI Labs.

7.28 Example 27: Blocking of MetQ substrate binding elicited by anti-lead candidate rabbit antisera

[00518] A competition assay by Surface Plasmon Resonance (SPR) was performed to assess the ability of the raised antibodies to block MetQ by blocking the binding to methionine (Met) and seleniomethionine (SeMet). Recombinant MetQ was immobilized onto flow cells of a CM5 Biosensor chip. Sera and Met or SeMet were flowed upon the immobilized protein or upon a no-protein control cell. Rabbit sera of Lead vaccine candidate (OMVs produced from the *E Coli.* strain of Example 13B), OMV containing only MetQ antigen (MetQ OMV) or control buffer

immunization were used as the first injection (single or in pools of 2 animals, 5-6 animals per group), and Met or SeMet as the second injection, with PBS used as a negative control. Binding of Met or SeMet to MetQ was analyzed in presence and absence of serum, and with pre and post-immune serum and run in duplicates or triplicates. Data was collected using the Pioneer Software package and analyzed using Qdat analysis software. The calculated blocking values are reported in **FIGS. 29A & 29B**. These results showed that the elicited antibodies against MetQ in post III sera from lead candidate and MetQ OMV were able to significantly block the binding of methionine (Met) or seleniomethionine (SeMet) to MetQ, compared to no sera, control buffer immunized sera or pre sera. Pre sera showed blocking activity compared to no sera control but the activity was significantly lower than Lead and MetQ OMV post-III immunization sera. **FIG. 29C** shows chemical structure of methionine and seleniomethionine.

7.29 Example 28: Blocking of MsrA/B substrate binding elicited by anti-lead candidate rabbit antisera

[00519] A competition assay by Surface Plasmon Resonance (SPR) was performed to assess the ability of the antibodies raised by immunizing rabbits with the lead candidate (OMVs produced from the *E Coli.* strain of Example 13B) to block MsrA/B by blocking the binding to oxidized methionine presented as methionine sulfoxide (MetSO) or oxidized peptide (Pep-O; VAPVGMHIANGM (SEQ ID NO: 69) from *N.gonorrhoeae* OMP). Methionine oxidation on the peptide was confirmed by Mass spectrometry. Before running the assay, non-oxidized peptide was tested for binding to MsrA/B as a control, and no binding was seen of MsrA/B to the reduced peptide. Recombinant MsrA/B was immobilized onto flow cells of a CM5 Biosensor chip. Sera and MetSO or Pep-O were flowed upon the immobilized protein or upon a no-protein control cell. Rabbit sera were used as the first injection, and MetSO or Pep-O as the second injection. Binding of MetSO or Pep-O to MsrA/B was analyzed in presence and absence of serum, and with pre- and post-immune serum. Data was collected using the Pioneer Software package and analyzed using Qdat analysis software. **FIGS 30A & 30B** show the calculated blocking values. These results showed that the elicited antibodies against MsrA/B in post III sera from lead vaccine candidate was able to significantly block binding of methionine sulfoxide and oxidized peptide to MsrA/B, compared to no sera control buffer immunized sera or pre sera.

7.30 Example 29: Blocking of *N. gonorrhoeae* adherence to cervical (ME180), pharyngeal (FaDu) and urethral (tUEC) epithelial cells mediated by anti-lead candidate rabbit antisera

[00520] Gonococcal adherence assays were performed to further assess the functionality of the elicited rabbit antibodies. Monolayers of human cervical epithelial cells (ME180), pharyngeal epithelial cells (FaDu), and urethral (tUEC) epithelial cells were pre-incubated with 1/200 diluted heat-inactivated rabbit sera (pools of 2 animals or single sera from 5 or 6 animals), then covered with *Neisseria gonorrhoeae* strain 1291 or *N. gonorrhoeae* ΔNHBA (where NHBA had been knocked out). Following the incubation, cell monolayers were washed three times to remove non-adherent bacteria, epithelial cells were lysed in 1% saponin (which did affect bacterial viability) and well contents were plated onto GC agar plates. Results were calculated as the mean CFU from three replicates and presented as percentage of adherent bacteria relative to non-treated adhesion. Statistical analysis was performed with ANOVA. **FIG. 31A** shows the percentage of adherent bacteria relative to non-treated adhesion for sera from rabbits immunized with buffer control, lead vaccine candidate (OMVs produced from the *E Coli.* strain of Example 13B), and OMV containing only NHBA antigen. Both *N. gonorrhoeae* strain 1291 and *N. gonorrhoeae* ΔNHBA were assayed using cervical epithelial cells (ME180) as monolayer. Lead vaccine candidate and NHBA OMV post-III sera significantly inhibit adherence of *N. gonorrhoeae* 1291 compared to control buffer immunized sera. However when *N. gonorrhoeae* ΔNHBA was used for adhesion, no sera showed adhesion inhibition, indicating that for ME180 cells, NHBA was involved in adhesion. **FIG. 31B** shows the percentage of adherent bacteria relative to non-treated adhesion for sera from rabbits immunized with buffer control, lead vaccine candidate, OMV containing only NHBA antigen (NHBA OMV, denoted as "NHBA" in **FIGS. 31A-31C**), and OMV containing only *N. gonorrhoeae* LOS antigen (NgLOS OMV, denoted as "NgLOS" in **FIG. 31B**). *N. gonorrhoeae* strain 1291 was assayed using pharyngeal epithelial cells (FaDu) as monolayer. Lead vaccine candidate, NHBA OMV and NgLOS OMV post-III sera significantly inhibited adherence of *N.gonorrhoeae* 1291 compared to control buffer immunized sera. **FIG. 31C** shows the percentage of adherent bacteria relative to non-treated adhesion for sera from rabbits immunized with buffer control, lead vaccine candidate and OMV containing only NHBA antigen (NHBA OMV). *N. gonorrhoeae* ΔNHBA was assayed using pharyngeal epithelial cells (FaDu) as monolayer. Lead vaccine candidate post-III sera significantly inhibited adherence of *N.gonorrhoeae* ΔNHBA compared to control buffer immunized sera and NHBA OMV immunized sera. When NHBA OMV post-III sera was used, no adhesion inhibition was shown. **FIGS 31B and 31C** indicated that, for

FaDu cells, NHBA was involved in adhesion, however other components of lead candidate sera were also able to inhibit adhesion. **FIG. 31D** shows the percentage of adherent bacteria relative to non-treated adhesion for sera from rabbits immunized with buffer control, lead vaccine candidate, OMV containing only NHBA antigen (NHBA OMV, denoted as "NHBA" in **FIGS. 31A-31D**), and OMV containing only *N. gonorrhoeae* LOS antigen (NgLOS OMV, denoted as "NgLOS" in **FIGS. 31B and 31D**). *N. gonorrhoeae* strain 1291 was assayed using urethral epithelial cells (tUEC) as monolayer. Lead vaccine candidate, NHBA OMV, and NgLOS OMV post-III sera significantly inhibited adherence of *N.gonorrhoeae* 1291 compared to control buffer immunized sera.

7.31 Example 30: Blocking of AccA binding to AniA mediated by anti-lead candidate rabbit antisera

[00521] AccA peptide binding inhibition to AniA. The assay was done to further assess the capacity of the elicited rabbit antibodies to bind AniA. AccA (AniA Cu chaperone A) donates Cu to the active site of AniA and is essential for AniA activity, if AccA is not bound to AniA, nitrite reductase activity is lost. The assays measured indirect inhibition of AniA activity. AccA peptide 17 (GSYHVMFMGLKKQLK (SEQ ID NO: 70)) corresponds to the Cu-binding site of AccA and can directly bind to AniA. Blocking binding of AccA peptide 17 inhibits nitrite reductase activity. A competition assay by Surface Plasmon Resonance (SPR) was performed to assess the ability of the antibodies raised by immunizing rabbits with the lead candidate (OMVs produced from the *E Coli.* strain of Example 13B) to block AniA by blocking the binding site to AccA peptide 17. Before running the assay, AccA peptide 17 was tested for binding to AniA as a control, and complete binding to AniA was seen. Recombinant AniA was immobilized onto flow cells of a CM5 Biosensor chip. Sera and AccA peptide 17 were flowed upon the immobilized protein. Rabbit sera were used as the first injection, and AccA peptide 17 as the second injection. Binding of AccA peptide 17 was analyzed in presence and absence of serum, and with pre- and post-immune serum of lead candidate vaccine and *E.coli* OMV carrying only AniA antigen (AniA OMV). Data was collected using the Pioneer Software package and analyzed using Qdat analysis software. **FIG. 33** shows the calculated blocking values. These results showed that the elicited antibodies against AniA in post III sera from lead vaccine candidate and AniA OMV were able to significantly block binding of AccA peptide 17 compared to no sera, control buffer immunized sera or pre sera.

7.32 Example 31: Immune responses elicited by Lead vaccine candidate and single-antigen OMV in mice

[00522] **Immunization**. To test the ability of the selected gonococcal antigen as monocomponet OMV and Lead vaccine configuration (OMVs produced from the *E Coli.* strain of

Example 13B) to elicit an immune response, immunization studies were carried out. BALB/c mice were immunized with OMV batches expressing i. AniA, ii. NHBA, iii. MsrA/B, iv. MetQ, v. NgLOS and 3 different batches of Lead vaccine (Lead batch 1, Lead CB1 and Lead CB2, where CB1 and CB2 were consistency batches) and buffer control group. All groups were immunized with alum as adsorbent. Each group consisted of 10 mice. The immunization schedule is reported in **FIG. 21A.**

[00523] **Immunogenicity**. **FIG. 34A** shows specific IgG titres (arbitrary units, AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0), and in terminal bleed sera ("post-III", at day 42). Pooled mice sera (for "pre") and individual mice sera (for "post-III") were measured by ECLIA (electrochemiluminescence immunoassay, Bolton JS *et al.*, *Malar J*, 2020;19(1):159) against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B. For the assay, biotinylated soluble protein antigens were used. Lead candidate (batch 1) and the two consistency batches (CB1 and CB2) had significantly higher immunogenicity at post-III than pre or control buffer immunization levels at post-III against the four proteins (AniA, MetQ, cNHBA, and MsrA/B). At post-III, single antigen OMVs had significantly higher immunogenicity against its own antigen compared to pre or control buffer immunization levels at post.III. **FIG. 34B** shows specific IgG titers (arbitrary units AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0), and in terminal bleed sera ("post-III", at day 42). Pooled pre immunization mice sera ("pre") and post-III individual mice sera ("post-III") were measured by ELISA (Enzyme-Linked Immunosorbent Assay) against NgLOS (produced in E.coli and purified containing both 2C7 and 6E4 epitopes). Lead candidate (batch 1) and the two consistency batches (CB1 and CB2) had significantly higher immunogenicity at post-III than pre or control buffer immunization levels at post-III against NgLOS. NgLOS OMVs had significantly higher immunogenicity against NgLOS antigen than pre or control buffer immunization levels at post.III

[00524] **Serum Bactericidal Activity (SBA).** To understand whether the single antigen OMV raised antibodies were able to trigger complement-mediated killing of *Neisseria gonorrhoeae*, a Luminescent Serum Bactericidal Activity (L-SBA) assay was used. Here, serial dilutions of sera obtained during mice immunization as described above were added to plates' wells containing human complement and log phase cultures of *Neisseria gonorrhoeae* strain FA1090 and of strain WHO-R and incubated. Cell viability was measured by mixing with BacTiter-Glo and luminescence reading on a microplate reader, and IC50 values (sera dilution needed for 50% killing) were calculated. Individual mice-sera after 3 immunizations (post-III) were analyzed. **FIG. 34C** shows that the lead candidate, single OMV containing either NHBA or NgLOS elicited

significant bactericidal sera against both FA1090 and NgWHO-R and that single OMVs containing AniA, MetQ or MsrA/B did not elicit significant bactericidal titers.

7.33 Example 32: Immune responses elicited by Lead candidate and single-antigen OMV in rabbit

[00525] **Immunization.** To test the ability of the selected gonococcal antigen as single OMV and Lead vaccine configuration (OMVs produced from the *E Coli.* strain of Example 13B) to elicit an immune response, immunization studies were carried out. New Zealand White rabbits were immunized with OMV batches expressing i. AniA, ii. NHBA, iii. MsrA/B, iv. MetQ, v. NgLOS and 2 different batches of Lead vaccine (Lead CB1 and Lead CB2 where CB1 and CB2 were consistency batches) and buffer control group. All groups were immunized with alum as adsorbent. Each group consisted of 5-6 rabbits. The immunization schedule is reported in **FIG. 21A** and is the same as used for mice.

[00526] **Immunogenicity. FIG. 35** shows specific IgG titres (arbitrary units, AU) in pre-immunization sera ("pre", or "Pre-bleed", at day 0), in sera drawn after the second immunization ("post-II", at day 28), and in terminal bleed sera ("post-III", at day 42). Individual rabbit sera (for "pre" and "post-III") or pooled sera ("post-II") were measured by ECLIA (electrochemiluminescence immunoassay, Bolton JS *et al., Malar J*, 2020;19(1):159) against each individual protein antigen AniA, MetQ, cNHBA and MsrA/B. For the assay, biotinylated soluble recombinant protein antigens were used. ELISA (Enzyme-Linked Immunosorbent Assay) was used to measure immune responses against NgLOS (which was produced in E.coli). Significant titer increase pre/post-III was elicited for all 5 antigens when Lead vaccine consistency batches were used. Single antigen OMV elicited significant increase in titer response against its own antigen at post-III vs pre.

7.34 Example 33: Immune responses elicited by Lead candidate in rat

[00527] **Immunization**. To test the ability of the selected gonococcal antigen to elicit an immune response when expressed on OMV, immunization studies were carried out. Sprague Dawley rats were immunized with 100 μg (in total protein) of Lead candidate vaccine (OMVs produced from the *E Coli.* strain of Example 13B) or with buffer control. Two groups were immunized with Lead candidate to test two different batches: lead batch and consistency batch 2 (CB2). All groups were immunized with alum as adsorbent. Vaccine groups consisted of 10 rats, and the control group consisted of 8 rats. The immunization schedule was the same as reported in **FIG. 21A** for mice.

[00528] **Immunogenicity. FIG. 36** shows endpoint titres of individual rats in pre-immunization sera ("pre", or "Pre-bleed", at day 0), in sera drawn after the second immunization ("post-II", at day 28), and in terminal bleed sera ("post-III", at day 42) against each individual protein antigen AniA, MetQ, cNHBA, MsrA/B and NgLOS (expressed in E.coli). Titers were measured by ELISA (Enzyme-Linked Immunosorbent Assay), where plates were coated soluble versions of the four protein antigens or NgLOS. All antigens elicited significantly higher titers at post-II and post-III than pre or buffer immunized group. Control buffer immunized group did not elicit immune responses against any antigen.

8. EQUIVALENTS

[00529] All patents and publications mentioned in this specification are incorporated herein by reference in their entireties. From the foregoing description, it will be apparent that variations and modifications can be made to the invention described herein to adopt it to various uses and conditions. Such embodiments are also within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An *Escherichia coli* (*E. coli*)-derived outer membrane vesicle (OMV) comprising a glycan antigen and a protein antigen of *N. gonorrhoeae*.

2. The OMV of claim 1, wherein the OMV further comprises a modified lipid A.

3. An *Escherichia coli* (*E. coli*)-derived outer membrane vesicle (OMV) comprising a glycan antigen and a protein antigen of a pathogen, and a modified lipid A.

4. The OMV of claim 3, wherein the pathogen is *N. gonorrhoeae*.

5. The OMV of any one of claims 2-4, wherein the modified lipid A is produced by at least one lipid A modification enzyme selected from the group consisting of msbB, pagL, lpxE, pagP, and combinations thereof.

6. The OMV of claim 5, wherein the msbB, pagL, and/or lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV.

7. The OMV of claim 5 or 6, wherein the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes.

8. The OMV of any one of claims 5-7, wherein the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

9. The OMV of any one of claims 2-8, wherein the modified lipid A comprises a mixture of heterogeneous lipid A species having different chemical structures.

10. The OMV of any one of claims 2-9, wherein the modified lipid A comprises at least one lipid A species comprising only one phosphate group.

11. The OMV of claim 10, wherein the modified lipid A comprises at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone.

12. The OMV of claim 11, wherein at least 60% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone.

13. The OMV of claim 11 or 12, wherein between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone.

14. The OMV of any one of claims 2-13, wherein the modified lipid A comprises at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone.

15. The OMV of claim 14, wherein at least 60% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone.

16. The OMV of claim 14 or 15, between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone.

17. The OMV of any one of claims 2-16, wherein the modified lipid A comprises at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A.

18. The OMV of claim 17, wherein at least 50% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A.

19. The OMV of claim 17 or 18, wherein between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A.

20. The OMV of any one of claims 2-19, wherein the modified lipid A comprises at least one lipid A species comprising at least three C14-3OH acyl chains.

21. The OMV of claim 20, wherein at least 60% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains.

22. The OMV of claim 20 or 21, wherein between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains.

23. The OMV of any one of claims 2-22, wherein the modified lipid A comprises at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone.

24. The OMV of claim 23, wherein at least 30% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone.

25. The OMV of claim 23 or 24, wherein between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone.

26. The OMV of any one of claims 2-25, wherein the modified lipid A comprises at least one lipid A species comprising a C12 acyl chain.

27. The OMV of claim 26, wherein at least 60% of the amount of the modified lipid A comprises a C12 acyl chain.

28. The OMV of claim 26 or 27, wherein between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

29. The OMV of any one of claims 1-28, wherein the protein antigen and glycan antigen are associated with the outer membrane of the OMV.

30. The OMV of any one of claims 1-29, wherein the protein antigen comprises a MsrA/B polypeptide.

31. The OMV of claim 30, wherein the MsrA/B polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 9.

32. The OMV of claim 30 or 31, wherein the MsrA/B polypeptide comprises the amino acid sequence of SEQ ID NO: 9.

33. The OMV of any one of claims 30-32, wherein the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine.

34. The OMV of claim 33, wherein the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14.

35. The OMV of claim 33 or 34, wherein the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14.

36. The OMV of any one of claims 1-35, wherein the protein antigen comprises a MetQ polypeptide.

37. The OMV of claim 36, wherein the MetQ polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7.

38. The OMV of claim 36 or 37, wherein the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7.

39. The OMV of any one of claims 1-38, wherein the protein antigen comprises an AniA polypeptide.

40. The OMV of claim 39, wherein the AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4.

41. The OMV of claim 39 or 40, wherein the AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

42. The OMV of any one of claims 1-38, wherein the protein antigen comprises a NHBA polypeptide.

43. The OMV of claim 42, wherein the NHBA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 16.

44. The OMV of claim 42 or 43, wherein the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16.

45. The OMV of any one of claims 42-44, wherein the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine.

46. The OMV of claim 45, wherein the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21.

47. The OMV of claim 45 or 46, wherein the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

48. An *E. coli*-derived OMV comprising an AniA polypeptide that is associated with the outer membrane of the OMV, optionally the OMV comprises a glycan antigen.

49. The OMV of claim 48, wherein the AniA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 4.

50. The OMV of claim 48 or 49, wherein the AniA polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

51. An *E. coli*-derived OMV comprising a MetQ polypeptide and a glycan antigen of a pathogen, wherein the MetQ polypeptide and the glycan antigen are associated with the outer membrane of the OMV.

52. The OMV of claim 51, wherein the MetQ polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 7.

53. The OMV of claim 51 or 52, wherein the MetQ polypeptide comprises the amino acid sequence of SEQ ID NO: 7.

54. An *E. coli*-derived OMV comprising a NHBA polypeptide and a glycan antigen of a pathogen, wherein the NHBA polypeptide and the glycan antigen are associated with the outer membrane of the OMV.

55. The OMV of claim 54, wherein the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine.

56. An *E. coli*-derived OMV comprising a NHBA polypeptide that is associated with the outer membrane of the OMV, wherein the N-terminus of the NHBA polypeptide is fused to the C-terminus of a lipidated MetQ polypeptide to form a NHBA-MetQ fusion polypeptide, wherein the lipidated MetQ polypeptide comprises a lipidated N-terminal cysteine.

57. The OMV of any one of claims 54-56, wherein the NHBA polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 16.

58. The OMV of any one of claims 54-57, wherein the NHBA polypeptide comprises the amino acid sequence of SEQ ID NO: 16.

59. The OMV of any one of claim 55-58, wherein the NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 21.

60. The OMV of any one of claim 55-59, wherein the NHBA-MetQ fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

61. An *E. coli*-derived OMV comprising a MsrA/B polypeptide and a glycan antigen of a pathogen, wherein the MsrA/B polypeptide and glycan antigen are associated with the outer membrane of the OMV.

62. The OMV of claim 61, wherein the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine.

63. An *E. coli*-derived OMV comprising a MsrA/B polypeptide that is associated with the outer membrane of the OMV, wherein the N-terminus of the MsrA/B polypeptide is fused to the C-terminus of a lipidated AniA polypeptide to form a MsrA/B-AniA fusion polypeptide, wherein the lipidated AniA polypeptide comprises a lipidated N-terminal cysteine.

64. The OMV of any one of claims 61-63, wherein the MsrA/B polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 9.

65. The OMV of any one of claims 61-64, wherein the MsrA/B polypeptide comprises the amino acid sequence of SEQ ID NO: 9.

66. The OMV of any one of claims 62-65, wherein the MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 90% sequence identity to the amino acid sequence of SEQ ID NO: 14.

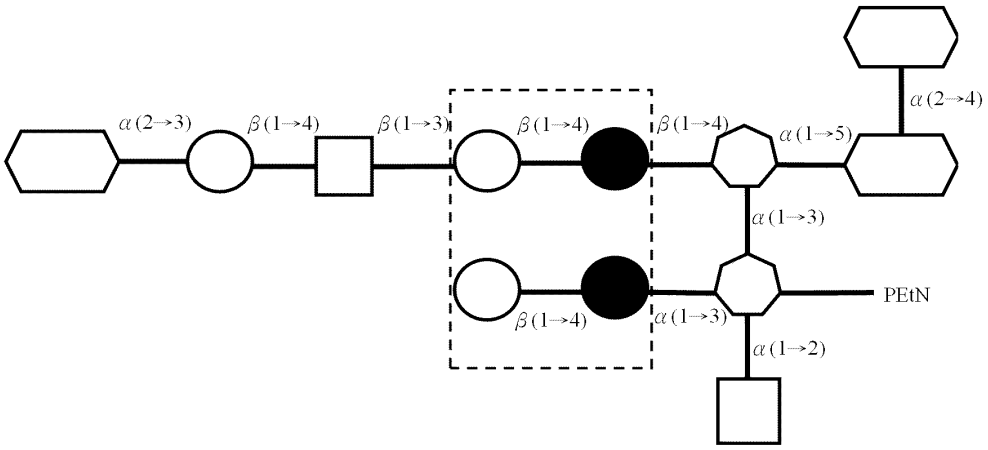
67. The OMV of any one of claims 62-66, wherein the MsrA/B-AniA fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 14.

68. The OMV of any one of the preceding claims, wherein the glycan antigen comprises keto-deoxyoctulosonate (KDO) as a terminal saccharide unit.

69. The OMV of claim 68, wherein glycan antigen comprises KDOα(2→3)Gal as the terminal saccharide unit.

70. The OMV of claim 68 or 69, wherein the glycan antigen comprises KDOα(2→3)Galβ(1→4)GlcNac as the terminal saccharide unit.

71. The OMV of any one of the preceding claims, wherein the glycan antigen comprises a 2C7 epitope having the structure within the broken line square of the following structure:

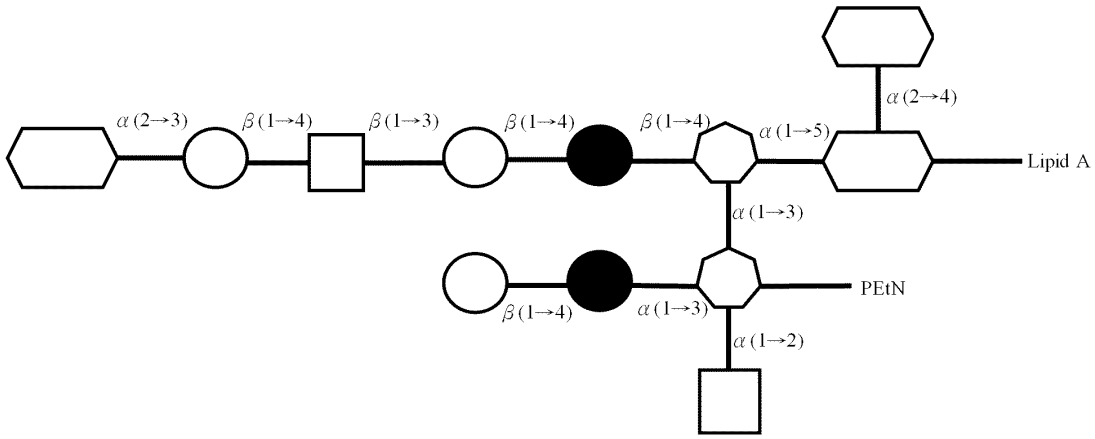


,

wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

72. The OMV of any one of the preceding claims, wherein the glycan antigen is covalently conjugated to the modified lipid A.

73. The OMV of claim 72, wherein the glycan antigen has the following structure:



, wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine.

74. An *E. coli*-derived OMV comprising:

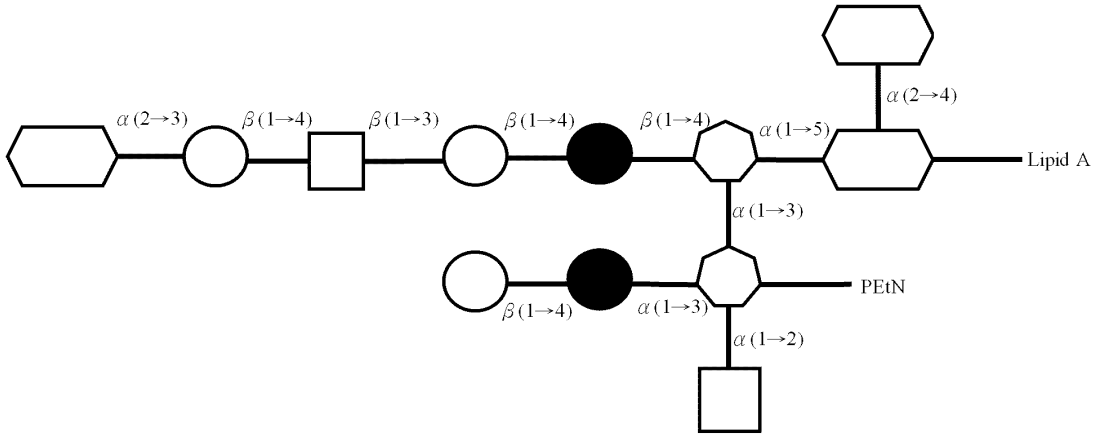
a. a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14;

b. a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7,

c. an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4,

d. a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 21, and

e. a glycan antigen has the following structure and is linked to a modified lipid A as follows:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced by a combination of msbB, pagL, lpxE, and pagP; wherein the msbB, pagL, and/or lpxE is encoded by at least one exogenous polynucleotide of an *E. coli* that produces the OMV, the *E. coli* comprises genetic disruption of *msbB*, *eptA*, and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

75. An *E. coli*-derived OMV comprising:

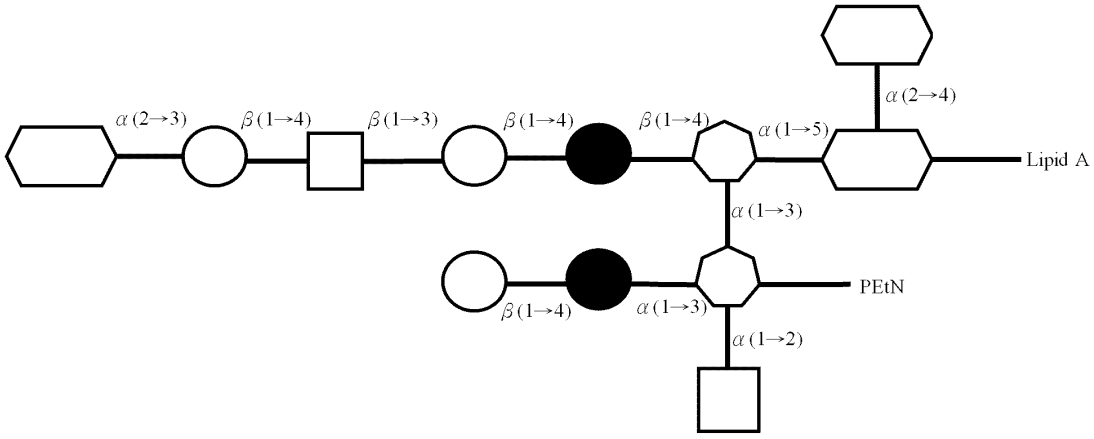
a. a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14;

b. a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7,

c. an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4,

d. a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 21, and

e. a glycan antigen has the following structure and is linked to a modified lipid A as follows:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A is produced a combination of pagL, lpxE, and pagP, wherein each of the pagL and lpxE is encoded by an exogenous polynucleotide of an *E. coli* that produces the OMV, wherein the *E. coli* comprises genetic disruption of *eptA* and *lpxT* genes, and the promoter of *pagP* gene of the *E. coli* is replaced by a lambda PL promoter.

76. An *E. coli*-derived OMV comprising:

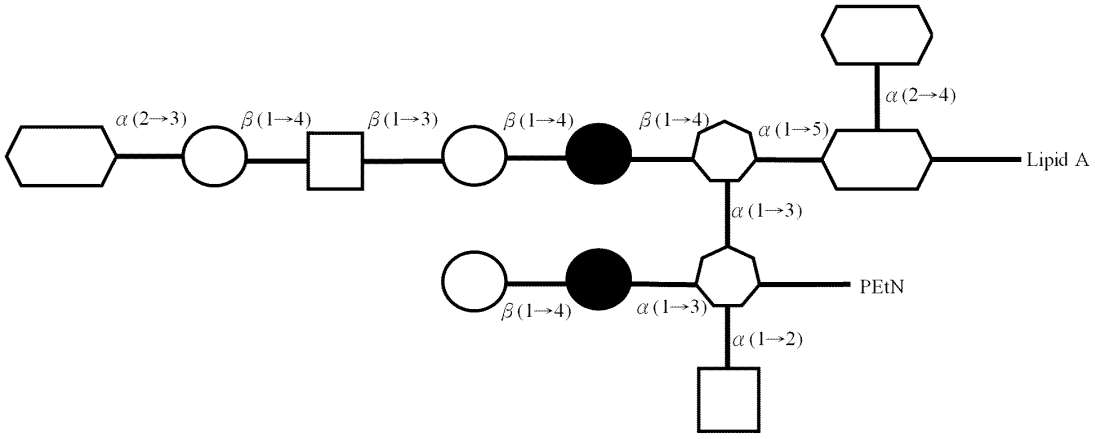
a. a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14;

b. a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7,

c. an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4,

d. a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 21, and

e. a glycan antigen has the following structure and is linked to a modified lipid A as follows:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine;

wherein the modified lipid A comprises (i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone; (ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide backbone; (iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains; (v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and (vi) at least one lipid A species comprising a C12 acyl chain.

77. An *E. coli*-derived OMV comprising:

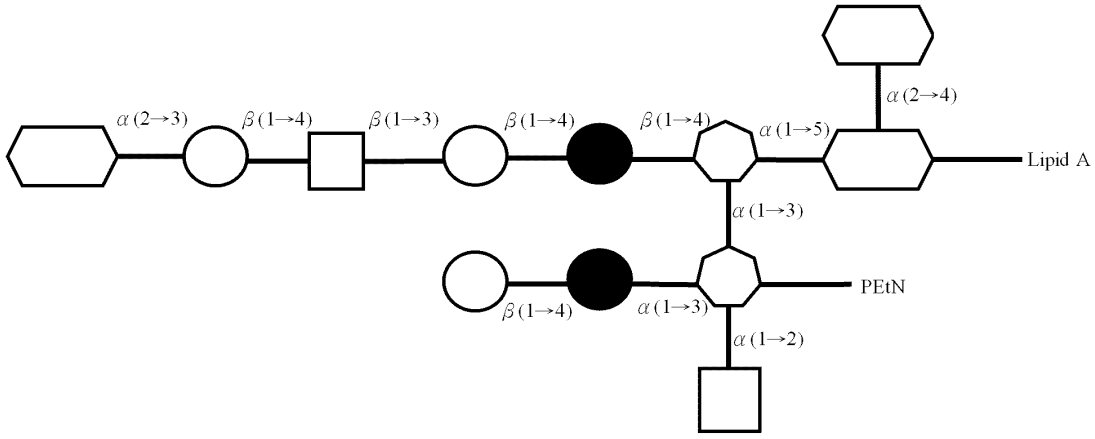
a. a MsrA/B-AniA fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 14;

b. a MetQ polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 7,

c. an AniA polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 4,

d. a NHBA-MetQ fusion polypeptide comprises an amino acid sequence having at least about 95% sequence identity to the amino acid sequence of SEQ ID NO: 21, and

e. a glycan antigen has the following structure and is linked to a modified lipid A as follows:



wherein the hexagon represents a KDO, the white circle represents a galactose (Gal), the white square represents a N- acetylglucosamine (GlcNac), the heptagon represents a L-glycero-D-manno-heptose (Hep), the black circle represents a glucose (Glc), and the PEtN represents a phosphoethanolamine; and

(i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone;

(ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone;

(iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A;

(iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains;

(v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and

(vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

78. The OMV of any one of claim 68-77, wherein the KDO can be recognized by antibody 6E4.

79. An *E. coli*-derived OMV comprising a modified lipid A, wherein the modified lipid A comprises

(i) at least one lipid A species comprising only one phosphate group attached at the 4' position of the disaccharide backbone;

(ii) at least one lipid A species lacking a fatty acyl chain at 3 position of the disaccharide

backbone;

(iii) at least one lipid A species that is 3-O-desacyl-4'-monophosphoryl lipid A; (iv) at least one lipid A species comprising at least three C14-3OH acyl chains;

(v) at least one lipid A species comprising a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and/or

(vi) at least one lipid A species comprising a C12 acyl chain.

80. An *E. coli*-derived OMV comprising a modified lipid A, wherein:

(i) between 70% and 75% of the amount of the modified lipid A comprises only one phosphate group attached at the 4' position of the disaccharide backbone;

(ii) between 80% and 90% of the amount of the modified lipid A lacks a fatty acyl chain at 3 position of the disaccharide backbone;

(iii) between 60% and 70% of the amount of the modified lipid A is 3-O-desacyl-4'-monophosphoryl lipid A;

(iv) between 90% and 100% of the amount of the modified lipid A comprises at least three C14-3OH acyl chains;

(v) between 40% and 50% of the amount of the modified lipid A comprises a C16 acyl chain that is linked to the C14-OH acyl chain at 2 position of the disaccharide backbone; and/or

(vi) between 90% and 100% of the amount of the modified lipid A comprises a C12 acyl chain.

81. A composition comprising the OMV of any one of claims 1-80.

82. A pharmaceutical composition comprising the OMV of any one of claims 1-80, and a pharmaceutically acceptable carrier.

83. An *Escherichia coli* engineered to produce the OMV of any one of claims 1-80.

84. The *E. coli* of claim 83, wherein the *E. coli* comprises one or more genetic modifications to express the protein antigen.

85. The *E. coli* of claim 84, wherein the *E. coli* comprises:

a. an exogenous polynucleotide encoding the MsrA/B polypeptide, optionally wherein the *E. coli* comprises an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and

an AniA signal polypeptide, further optionally wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

b. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, optionally wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

c. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, optionally wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and/or

d. an exogenous polynucleotide encoding the NHBA polypeptide, optionally wherein the *E. coli* comprises an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, further optionally wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

86. The *E. coli* of any one of claims 83-85, wherein the *E. coli* comprises one or more genetic modifications to produce the modified lipid A.

87. The *E. coli* of claim 86, wherein the *E. coli* comprises:

a. an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and/or an exogenous polynucleotide encoding lpxE;

b. genetic disruption of *msbB*, *eptA*, and/or *lpxT* genes; and/or

c. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter.

88. The *E. coli* of any one of claims 83-87, wherein the *E. coli* comprises one or more genetic modifications to produce the glycan antigen.

89. The *E. coli* of claim 88, wherein the *E. coli* comprises:

a. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and/or an exogenous polynucleotide encoding kdsB;

b. genetic disruption of *waaQ*, *waaG*, *waaP*, *waaS*, *waaB*, *waaO*, *waaR*, *waaY*, *waaZ*, and/or *waaU* genes;

c. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and/or *'wbbL* genes; and/or

d. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and/or *wecG* genes.

90. The *E. coli* of any one of claims 83-89, wherein the *E. coli* further comprises one or more genetic modifications to increase vesiculation.

91. The *E. coli* of claim 90, wherein the *E. coli* further comprises:

a. genetic disruption of *ompA* gene;

b. genetic disruption of *lpp* gene; and/or

c. an exogenous polynucleotide encoding lpp.

92. The *E. coli* of any one of claims 83-91, wherein the *E. coli* further comprises genetic disruption of *fliC* gene, optionally wherein the *E. coli* further comprises genetic disruption of *fim* gene cluster, further optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

93. An *Escherichia coli* (*E. coli*) engineered to produce an OMV, wherein the *E. coli* comprises:

a. genetic modifications to express the protein antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

ii. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

iii. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and

iv. an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

b. genetic modifications to produce the modified lipid A, wherein the *E. coli* comprises:
    i. an exogenous polynucleotide encoding msbB, an exogenous polynucleotide encoding pagL, and an exogenous polynucleotide encoding lpxE;
    ii. genetic disruption of *msbB, eptA,* and *lpxT* genes; and
    iii. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;
c. genetic modifications to produce the glycan antigen, wherein the *E. coli* comprises:
    i. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB;
    ii. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes;
    iii. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL*; and
    iv. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;
d. genetic modifications to increase vesiculation, wherein the *E. coli* comprises:
    i. genetic disruption of *ompA* and *lpp* genes;
    ii. an exogenous polynucleotide encoding lpp, optionally wherein the exogenous polynucleotide encoding lpp is inserted at the attλ site of the genome of the *E. coli* under the promoter Para;
e. genetic disruption of *fliC* gene; and

f. genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

94. An *Escherichia coli* (*E. coli*) engineered to produce an OMV, wherein the *E. coli* comprises:

a. genetic modifications to express the protein antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3;

ii. an exogenous polynucleotide encoding the MetQ polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

iii. an exogenous polynucleotide encoding the AniA polypeptide and an AniA signal polypeptide, wherein the AniA signal polypeptide comprises the amino acid sequence of SEQ ID NO: 3; and

iv. an exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and a MetQ signal polypeptide, wherein the MetQ signal polypeptide comprises the amino acid sequence of SEQ ID NO: 6;

b. genetic modifications to produce the modified lipid A, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding pagL and an exogenous polynucleotide encoding lpxE;

ii. genetic disruption of *eptA* and *lpxT* genes; and

iii. the promoter of *pagP* gene of the *E. coli* being replaced by a lambda PL promoter;

c. genetic modifications to produce the glycan antigen, wherein the *E. coli* comprises:

i. an exogenous polynucleotide encoding lpt3, an exogenous polynucleotide encoding rfaK, an exogenous polynucleotide encoding lgtF, an exogenous polynucleotide encoding lgtG, an exogenous polynucleotide encoding lgtE, an exogenous polynucleotide encoding lgtA, an exogenous polynucleotide encoding lgtB, an exogenous

polynucleotide encoding lst, and an exogenous polynucleotide encoding kdsB;

ii. genetic disruption of *waaQ, waaG, waaP, waaS, waaB, waaO, waaR, waaY, waaZ,* and *waaU* genes;

iii. genetic disruption of *wza, wzb, wzc, wcaA, wcaB, wcaC, wcaD, wcaE, wcaF, gmd, fcl, gmm, wcaI, cpsB, cpsG, wcaJ, wzxC, wcaK, wcaL, wcaM, galF, rfbB, rfbD, rfbA, rfbC, wzx, glf, wzy, wbbI, wbbJ, wbbL', intS,* and *'wbbL*; and

iv. genetic disruption of *wecA, wzzE, wecB, wecC, rmlB, rmlA, wecD, wecE, wzxE, wecF, wzyE,* and *wecG* genes;

d. genetic modifications to increase vesiculation, wherein the *E. coli* comprises:

i. genetic disruption of *ompA* and *lpp* genes;

ii. an exogenous polynucleotide encoding lpp, optionally wherein the exogenous polynucleotide encoding lpp is inserted at the attλ site of the genome of the *E. coli* under the promoter Para;

e. genetic disruption of *fliC* gene; and

f. genetic disruption of *fim* gene cluster, optionally wherein the *fim* gene cluster comprises *fimHGFDCIA* genes.

95. The *E. coli* of claim 93 or 94, wherein the exogenous polynucleotide encoding the MsrA/B-AniA fusion polypeptide and the AniA signal polypeptide, the exogenous polynucleotide encoding the MetQ polypeptide and the MetQ signal polypeptide, the exogenous polynucleotide encoding the AniA polypeptide and the AniA signal polypeptide, the exogenous polynucleotide encoding the NHBA-MetQ fusion polypeptide and the MetQ signal polypeptide, and the exogenous polynucleotide encoding KdsB are expressed from a first vector.

96. The *E. coli* of any one of claims 93-95, wherein the exogenous polynucleotide encoding lst is expressed from a second vector, wherein the expression of lst is constitutively under control of lambda phage promoter PL in the second vector.

97. The *E. coli* of any one of claims 93-96, wherein the exogenous polynucleotide encoding msbB, the exogenous polynucleotide encoding pagL, the exogenous polynucleotide encoding lpxE, the exogenous polynucleotide encoding lpt3, the exogenous polynucleotide encoding rfaK, the exogenous polynucleotide encoding lgtF, the exogenous polynucleotide encoding lgtG, the

exogenous polynucleotide encoding lgtE, the exogenous polynucleotide encoding lgtA, the exogenous polynucleotide encoding lgtB, the exogenous polynucleotide encoding lst, and the exogenous polynucleotide encoding lpp are incorporated into the genome of the *E. coli*.

98. A method of producing a plurality of OMVs, comprising (i) culturing the *E. coli* of any one of claims 83-97 under conditions suitable for producing OMVs, and (ii) purifying the OMVs produced from the *E. coli*.

99. A method of inducing an immune response in a subject against *N. gonorrhoeae* comprising administering to the subject an effective amount of the OMV of any one of claims 1-80 or the composition of claim 81 or 82.