





The Lst Sialyltransferase of *Neisseria gonorrhoeae* Can Transfer Keto-Deoxyoctanoate as the Terminal Sugar of Lipooligosaccharide: a Glyco-Achilles Heel That Provides a New Strategy for Vaccines to Prevent Gonorrhea

Freda E.-C. Jen,^a Margaret R. Ketterer,^b Evgeny A. Semchenko,^a Christopher J. Day,^a  Kate L. Seib,^a Michael A. Apicella,^b  Michael P. Jennings^a

^aGriffith University, Institute for Glycobiology, Gold Coast, Queensland, Australia

^bDepartment of Microbiology and Immunology and Department of Internal Medicine, The University of Iowa, Iowa City, Iowa, USA

ABSTRACT The lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* plays key roles in pathogenesis and is composed of multiple possible glycoforms. These glycoforms are generated by the process of phase variation and by differences in the glycosyltransferase gene content of particular strains. LOS glycoforms of *N. gonorrhoeae* can be terminated with an *N*-acetylneuraminic acid (Neu5Ac), which imparts resistance to the bactericidal activity of serum. However, *N. gonorrhoeae* cannot synthesize the CMP-Neu5Ac required for LOS biosynthesis and must acquire it from the host. In contrast, *Neisseria meningitidis* can synthesize endogenous CMP-Neu5Ac, the donor molecule for Neu5Ac, which is a component of some meningococcal capsule structures. Both species have an almost identical LOS sialyltransferase, Lst, that transfers Neu5Ac from CMP-Neu5Ac to the terminus of LOS. Lst is homologous to the LsgB sialyltransferase of nontypeable *Haemophilus influenzae* (NTHi). Studies in NTHi have demonstrated that LsgB can transfer keto-deoxyoctanoate (KDO) from CMP-KDO to the terminus of LOS in place of Neu5Ac. Here, we show that Lst can also transfer KDO to LOS in place of Neu5Ac in both *N. gonorrhoeae* and *N. meningitidis*. Consistent with access to the pool of CMP-KDO in the cytoplasm, we present data indicating that Lst is localized in the cytoplasm. Lst has previously been reported to be localized on the outer membrane. We also demonstrate that KDO is expressed as a terminal LOS structure *in vivo* in samples from infected women and further show that the anti-KDO monoclonal antibody 6E4 can mediate opsonophagocytic killing of *N. gonorrhoeae*. Taken together, these studies indicate that KDO expressed on gonococcal LOS represents a new antigen for the development of vaccines against gonorrhea.

IMPORTANCE The emergence of multidrug-resistant *N. gonorrhoeae* strains that are resistant to available antimicrobials is a current health emergency, and no vaccine is available to prevent gonococcal infection. Lipooligosaccharide (LOS) is one of the major virulence factors of *N. gonorrhoeae*. The sialic acid *N*-acetylneuraminic acid (Neu5Ac) is present as the terminal glycan on LOS in *N. gonorrhoeae*. In this study, we made an unexpected discovery that KDO can be incorporated as the terminal glycan on LOS of *N. gonorrhoeae* by the alpha-2,3-sialyltransferase Lst. We showed that *N. gonorrhoeae* express KDO on LOS *in vivo* and that the KDO-specific monoclonal antibody 6E4 can direct opsonophagocytic killing of *N. gonorrhoeae*. These data support further development of KDO-LOS structures as vaccine antigens for the prevention of infection by *N. gonorrhoeae*.

KEYWORDS *Neisseria gonorrhoea*, lipooligosaccharide (LOS), keto-deoxyoctanoate (KDO), gonococcal vaccine, *N*-acetylneuraminic acid (Neu5Ac), sialic acid, Lst sialyltransferase

Citation Jen FEC, Ketterer MR, Semchenko EA, Day CJ, Seib KL, Apicella MA, Jennings MP. 2021. The Lst sialyltransferase of *Neisseria gonorrhoeae* can transfer Keto-Deoxyoctanoate as the terminal sugar of lipooligosaccharide: a glyco-Achilles heel that provides a new strategy for vaccines to prevent gonorrhea. mBio 12:e03666-20. <https://doi.org/10.1128/mBio.03666-20>.

Editor Larry S. McDaniel, University of Mississippi Medical Center

Copyright © 2021 Jen et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Michael A. Apicella, michael-apicella@uiowa.edu, or Michael P. Jennings, m.jennings@griffith.edu.au.

This article is a direct contribution from Michael A. Apicella, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by William Shafer, Emory University School of Medicine, and Sanjay Ram, University of Massachusetts Medical School.

Received 5 January 2021

Accepted 7 January 2021

Published 23 March 2021

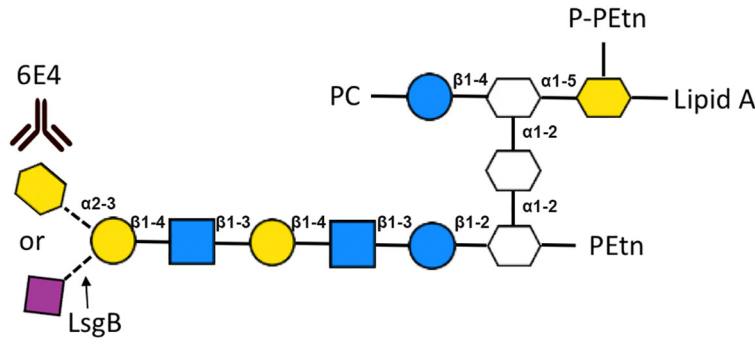
N*isseria gonorrhoeae* is a host-adapted bacterial pathogen that causes the sexually transmitted disease gonorrhea. Gonococcal infections can be symptomatic or asymptomatic, with up to 80% of infections in females being asymptomatic (1, 2). Untreated gonorrhea in females can lead to pelvic inflammatory disease (3), adverse pregnancy outcomes, neonatal complications, or infertility (4). Moreover, it can increase the risk of acquiring and transmitting HIV (5). It is estimated that there are more than 106 million cases of gonorrhea worldwide each year (6), and the emergence of multidrug-resistant (MDR) strains of *N. gonorrhoeae* is a major public health problem (7, 8). Isolates with resistance to nearly every antibiotic used to treat gonorrhea have been identified (9–12). In the past century, only three potential gonococcal vaccines have been tested in clinical trials, and none of these vaccines were successful (13–15). As such, there is an urgent unmet need for novel approaches for therapeutic and vaccine development.

Lipooligosaccharide (LOS) is one of the major structural components of the outer membrane and is a key virulence factor of *N. gonorrhoeae* (16). It plays a number of key roles in the pathogenesis of *N. gonorrhoeae*, including mediating direct interaction between *N. gonorrhoeae* and human urethral epithelial cells (17, 18). The lipid A of LOS of *N. gonorrhoeae* binds to complement component C3, which is required for activation of complement receptor 3 in primary cervical epithelial cells (19). The sugar composition of LOS of *N. gonorrhoeae* is variable due to phase variation (high frequency of on/off switching of expression) of the genes involved in LOS biosynthesis (20–22). The LOS structure of *N. gonorrhoeae* can be terminated with an *N*-acetylneuraminic acid (Neu5Ac) (Fig. 1) (23–25), which has multiple roles in gonococcal virulence (reviewed in reference 26). However, *N. gonorrhoeae* cannot synthesize the nucleotide sugar precursor, cytidine-5'-monophosphate (CMP)-Neu5Ac, that is required for LOS biosynthesis and must acquire CMP-Neu5Ac from the host (24). In contrast, the closely related pathogen *Neisseria meningitidis* can synthesize CMP-Neu5Ac, which is also required for the polysaccharide capsule structure of meningococcal serogroups B, C, W, and Y (27). Both of these pathogenic *Neisseria* species have an almost identical (95% amino acid identity) sialyltransferase, Lst, that transfers Neu5Ac from CMP-Neu5Ac to the terminal galactose of LOS (Fig. 1A). The Lst sialyltransferase expressed by the pathogenic *Neisseria* species is homologous to the LsgB sialyltransferase of nontypeable *Haemophilus influenzae* (NTHi) (28, 29). Recent studies in NTHi have demonstrated that the LsgB sialyltransferase can transfer keto-deoxyoctanoate (KDO) from CMP-KDO to LOS in place of Neu5Ac (29) (Fig. 1A). Recent studies in *N. gonorrhoeae* have demonstrated that Lst can transfer non-Neu5Ac substrates to LOS (30, 31). In this study, we investigated whether KDO can also be incorporated as the terminal glycan on LOS of *N. gonorrhoeae* by Lst, which has significant implications for our understanding of sialic acid biology and gonococcal vaccine development.

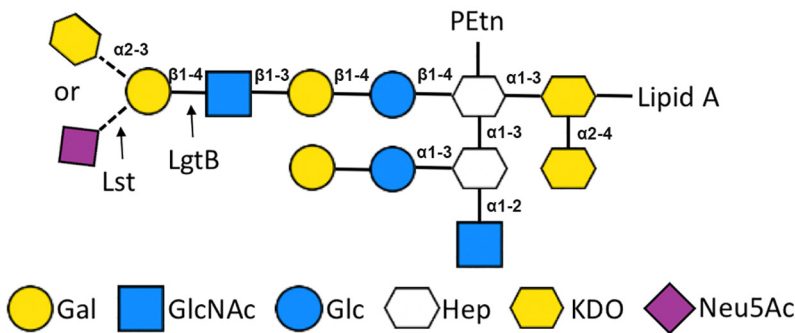
RESULTS

The *Neisserial* Lst sialyltransferase can add KDO to gonococcal LOS. NTHi has four sialyltransferases (SiaA, LsgB, Lic3A, and Lic3B) (32, 33), while *N. gonorrhoeae* has only one sialyltransferase, Lst (34). BLAST analysis indicated that Lst belongs to the glycosyltransferase superfamily 52 and is homologous to the sialyltransferase LsgB in NTHi (27% identity at the amino acid level, *e* value of $6e^{-31}$). To determine whether *N. gonorrhoeae* can transfer KDO as the terminal monosaccharide of LOS (29), similar to NTHi, whole-cell enzyme-linked immunosorbent assay (ELISA) was performed with the LOS terminal KDO-specific monoclonal antibody (MAb) 6E4 (29) on three well-characterized *N. gonorrhoeae* strains, 1291, MS11, and F62, and their *lst* knockout mutants. All three wild-type strains were KDO positive, while none of the *lst* mutants of these strains reacted with MAb 6E4 (Fig. 2A), indicating that Lst is required for KDO transfer to LOS. Consistent with previous studies, 6E4 cannot detect the basal KDO residue that is common to all LOS structures (29). Further analysis detected KDO (Fig. 2B) in 19 of 20 gonococcal clinical isolates from patients with mucosal or disseminated gonococcal

A Nontypeable *H. influenzae* LOS



***N. gonorrhoeae* 1291 LOS**



● Gal ■ GlcNAc ● Glc ◻ Hep ● KDO ◆ Neu5Ac

B

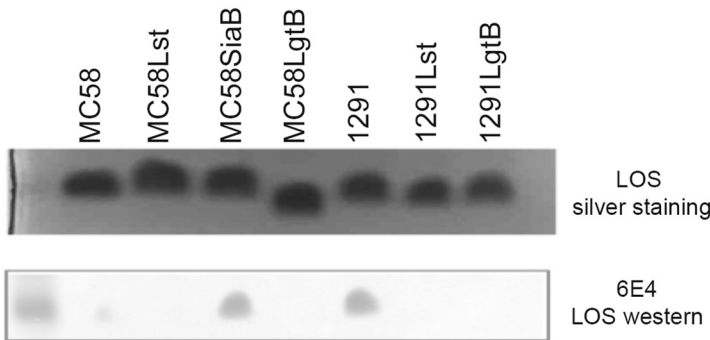


FIG 1 (A) Structure of the KDO-containing LOS of NTHi and *N. gonorrhoeae*. MAb 6E4 only recognizes the terminal KDO glycan. Lst and LsgB are the sialyltransferases that can also incorporate KDO onto the terminal Gal in the absence of Neu5Ac. LgtB is the galactosyltransferase that transfers the KDO acceptor galactose (Gal) to *N*-acetylglucosamine (GlcNAc). (B) Silver staining and 6E4 Western blot of purified LOS samples from MC58, 1291 wild-type, and glycotransferase mutant strains.

infections (35). Variable presentation of KDO on gonococcal LOS may be due to the absence of a terminal galactose acceptor if LgtA is switched off (Fig. 1) or via competition if another glycosyltransferase, LgtD, is phase varied “on” and transfers GalNAc in competition with Lst/CMP-KDO for the terminal galactose acceptor. The transfer of KDO to LOS was also investigated in *N. meningitidis*, which, unlike *N. gonorrhoeae*, can synthesize CMP-Neu5Ac via SiaB (27, 36). The ELISA results in Fig. 2C show the absence of KDO in the meningococcal strain MC58 wild type and *lst* mutant. However, an MC58 *siaB* mutant, which cannot synthesize CMP-Neu5Ac, was recognized by MAb 6E4, indicating the presence of KDO. These data indicate that there is competition between the CMP-Neu5Ac and CMP-KDO substrates for Lst-mediated transfer of the terminal sugar to LOS and that a mutation in *N. meningitidis* that ablates CMP-Neu5Ac synthesis is

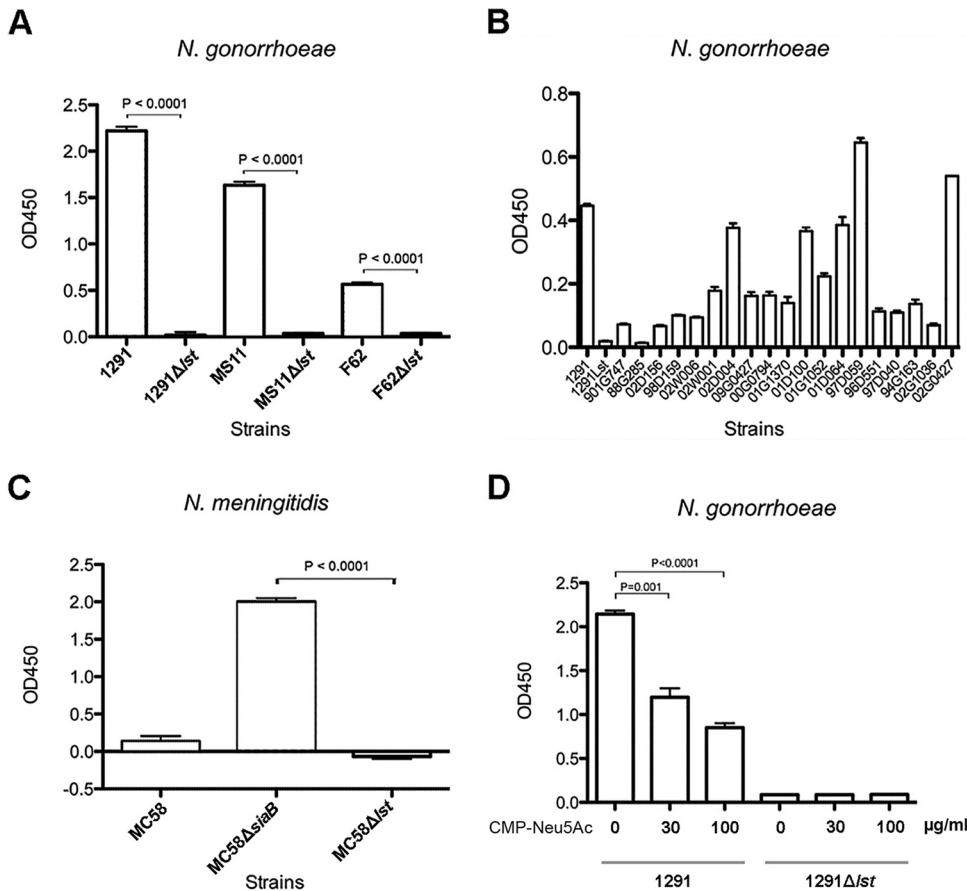


FIG 2 Whole-cell ELISA of 6E4. (A) ELISA of whole-cell *N. gonorrhoeae* WT and *Ist* mutant strains using 6E4 MAb. *Ist* transfers the terminal sialic acid to the terminal Gal of LOS in *N. meningitidis*. Here, the *Ist* mutant showed *Ist* also transfers KDO to the terminal Gal in *N. gonorrhoeae*. (B) Whole-cell ELISA of 6E4 was performed to test 20 clinical *N. gonorrhoeae* isolates. 1291 and 1291Lst were the positive and negative controls in this ELISA. (C) ELISA of whole-cell *Neisseria meningitidis* MC58 WT, *SiaB*, and *Ist* mutant strains using 6E4 MAb. MC58 WT has Neu5Ac as the terminal glycan of LOS. Without *SiaB*, *N. meningitidis* cannot make CMP-Neu5Ac; therefore, *Ist* adds KDO at the terminal of LOS instead of sialic acid. (D) Incorporation of KDO on gonococcal LOS. Whole-cell ELISA of 6E4 showed that the presence of CMP-Neu5Ac in the growing medium could inhibit the incorporation of KDO into the terminal Gal. *N. gonorrhoeae* 1291 and *Ist* mutant were grown in 0, 30 mM, and 100 μg/ml CMP-Neu5Ac supplemented media.

sufficient to permit KDO addition to LOS. To define the terminal KDO structure on the LOS, MAb 6E4 Western blotting with LOS purified from MC58, 1291, and their LOS glycotransferase (*Ist* and *IgtB*) mutant strains was performed. *IgtB* is a β1,4-galactosyltransferase that transfers CMP-Neu5Ac and CMP-KDO receptor galactose (Gal) to the *N*-acetylglucosamine (GlcNAc). As shown in Fig. 1B, the purified LOS from the 1291 wild type and the MC58 *siaB* mutant was 6E4 positive, while the 1291 and MC58 *Ist* and *IgtB* mutants all were 6E4 negative. LOS from the MC58 wild type was slightly 6E4 positive, indicating the presence of a small amount of KDO incorporated at the terminal on LOS.

CMP-Neu5Ac competes with KDO for addition to *N. gonorrhoeae* LOS by *Ist*.

The gonococcus cannot synthesize CMP-Neu5Ac as a substrate for LOS sialylation (37) due to a lack of CMP-Neu5Ac synthetase. To determine whether the incremental addition of CMP-Neu5Ac to the growth media can compete with CMP-KDO for *Ist* addition to LOS and thereby reduced binding of MAb 6E4, *N. gonorrhoeae* strain 1291 was grown in 0, 30 μg/ml, and 100 μg/ml CMP-Neu5Ac and whole-cell ELISA was performed. The results in Fig. 2D show that dose-dependent inhibition of 6E4 MAb binding to whole cells begins at 30 μg/ml CMP-Neu5Ac and is increased at 100 μg/ml.

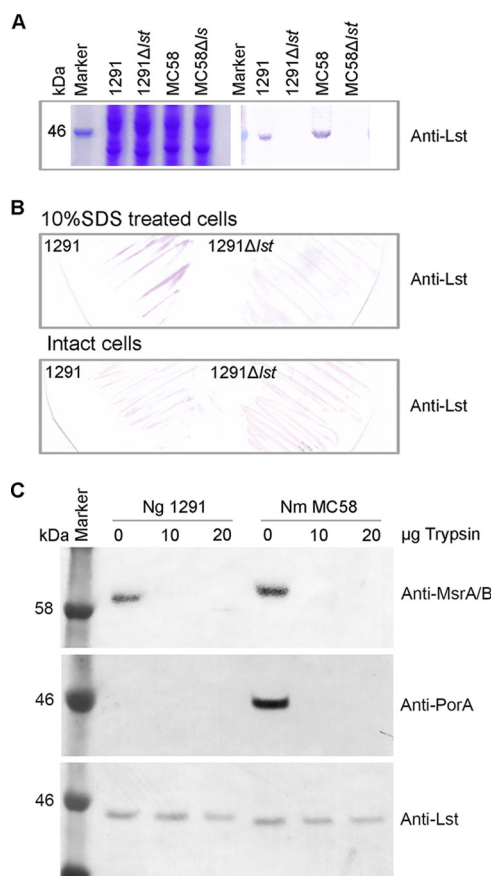


FIG 3 Cellular localization of Lst. (A) Western blot analysis of anti-Lst polyclonal antisera binding to Lst in whole-cell lysates. Coomassie staining gel showed equal loading of cell lysates. (B) Strain 1291 wild type and *lst* mutant were blotted on the membrane. Cells were treated with or without 10% SDS. Anti-Lst could detect Lst only when the cell was lysed. (C) Western blot analysis of whole, intact *N. gonorrhoeae* 1291 and *N. meningitidis* MC58 cells treated with increasing concentrations of trypsin (0, 10, and 20 μg) and probing with anti-Lst serum. Published neisserial MsrAB protein and the meningococcal PorA protein are the controls of surface protein.

These data suggest that CMP-Neu5Ac taken up from the media competes with endogenous CMP-KDO as a substrate for Lst activity.

Lst is localized in the cytoplasm of pathogenic *Neisseria*. The LOS core oligosaccharide is assembled on the inner face of the cytoplasmic membrane (38–40). The glycosyl transferases associated with LOS biosynthesis are localized in the cytoplasm, loosely associated with the membrane (reviewed in reference 38). Previously, the alpha-2,3-sialyltransferase Lst of *N. gonorrhoeae* was reported to be a surface-exposed outer membrane protein that could be a vaccine candidate (41). However, in the current study we demonstrate that KDO can be added to LOS in an Lst-dependent fashion. Furthermore, it is well established that CMP-KDO biosynthesis and utilization occur in the cytoplasm and that it has a very short half-life and a fast decomposition rate (42), indicating that Lst utilization of KDO must occur in the cytoplasm. Therefore, we performed a detailed investigation of the cellular location of Lst. First, SignalP (43) analysis predicts that Lst contains no N-terminal signal peptide, suggesting that it is not a secreted protein. Second, recombinant His-tagged Lst protein was expressed and purified and mouse polyclonal anti-Lst serum was produced. The anti-Lst serum bound specifically to the Lst protein in *N. gonorrhoeae* and *N. meningitidis* wild-type cell lysates in the Western blot analysis (Fig. 3A). Whole-cell colony blots revealed that the anti-Lst serum could only detect Lst when the cells were lysed by 10% SDS (Fig. 3B). Furthermore, trypsin digestion of surface-exposed proteins shows detection of Lst in

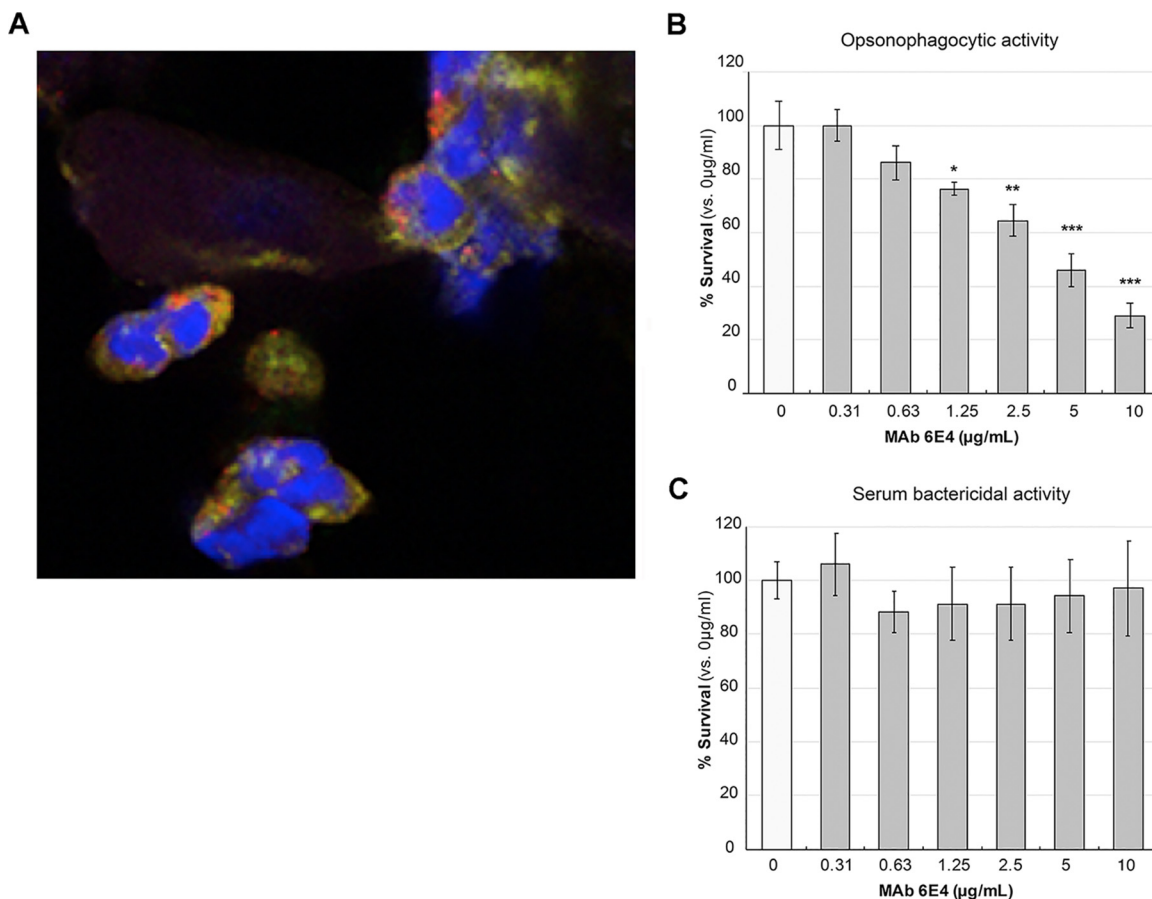


FIG 4 (A) Confocal microscopy study of cervical swab from a patient with a documented *N. gonorrhoeae* infection stained with both anti-KDO MAb 6E4 (Texas red) and anti-LOS MAb 6B4 (FITC). Infected PMNs can be seen with gonococci stained with both antibodies separately (red or green) and with antibodies colocalized to the same organisms (yellow). This study indicates that KDO termination occurs *in vivo* as well as *in vitro*. (B) Opsonophagocytic activity of MAb 6E4 against *N. gonorrhoeae* strain 1291. The survival of *N. gonorrhoeae* in the presence of 0 to 10 μg/ml MAb 6E4, primary human PMNs, and 10% normal human serum as a complement source is shown. Data represent the average survival (± 1 standard deviation) for triplicate samples, shown as a percentage of bacteria in the absence of antibody (0 μg/ml MAb 6E4, set at 100%, represents 5.07×10^3 CFU). There was a statistically significant difference between groups, as determined by one-way ANOVA [$F(6,14) = 59.20$, $P < 0.0001$]. Statistically significant differences relative to the untreated wild type, using a two-tailed Student's *t* test, are indicated: *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (*P* values were the following: 1.25 μg/ml, 0.012; 2.5 μg/ml, 0.005; 5 μg/ml, 0.001; and 10 μg/ml, 0.0003.) (C) Serum bactericidal activity. The survival of *N. gonorrhoeae* in the presence of 0 to 10 μg/ml MAb 6E4 and 10% normal human serum is shown. Data represent the mean survival (± 1 standard deviation) for triplicate samples as a percentage of bacteria in the absence of antibody (0 μg MAb 6E4, set at 100%, represents 4.53×10^3 CFU). There was no statistically significant difference between group means, as determined by one-way ANOVA [$F(6,14) = 0.71$, $P = 0.65$]. There was no statistically significant difference for any group relative to the untreated wild type, as determined using a two-tailed Student's *t* test ($P \geq 0.12$).

whole-cell *N. gonorrhoeae* and *N. meningitidis* treated with 0, 10, and 20 μg of trypsin for 60 min (Fig. 3C). Lst was not digested by trypsin. However, the known outer membrane surface proteins MsrAB and PorA were both digested by the trypsin concentrations tested. CFU counts between pre- and post-trypsin treatment were measured to ensure trypsin digestion of the bacterial surface did not affect cell viability (data not shown). Together, these data indicate that Lst is located in the cytoplasm, while none of the data support localization of Lst on the surface of the outer membrane.

The terminal KDO epitope is present in *N. gonorrhoeae* *in vivo*. To examine if *N. gonorrhoeae* can incorporate KDO as the terminal glycan of LOS during *in vivo* infection, a confocal microscopy study was conducted using MAb 6E4 on the cervical swab from a patient with a documented *N. gonorrhoeae* infection. Figure 4A demonstrates that *in vivo*, the terminal KDO epitope is present in *N. gonorrhoeae* on cervical epithelial cells. This suggests that the free CMP-Neu5Ac in the cervical environment was not sufficient to inhibit Lst incorporating KDO into LOS.

MAb 6E4 mediates opsonophagocytic killing. KDO is a sugar that is not found in humans, and studies in *H. influenzae* have proposed KDO as a vaccine antigen (1). To examine if the surface-expressed KDO in *N. gonorrhoeae* could be a vaccine target, MAb 6E4 was used in opsonophagocytic killing and serum bactericidal assays against *N. gonorrhoeae* strain 1291. Dose-dependent opsonophagocytic killing was observed from 0.65 to 10 $\mu\text{g/ml}$ MAb 6E4, with 5 $\mu\text{g/ml}$ MAb 6E4 eliciting 54% killing and 10 $\mu\text{g/ml}$ eliciting 71% killing relative to the no treatment control (Fig. 4B). No complement-dependent serum bactericidal activity was observed at concentrations up to 10 $\mu\text{g/ml}$ MAb 6E4 (Fig. 4C). These data suggest that antibodies targeting KDO can mediate bacterial killing via opsonophagocytic activity and that the LOS terminal KDO epitope in *N. gonorrhoeae* is a potential vaccine target.

DISCUSSION

N. gonorrhoeae is an obligate human pathogen that is highly adapted to the human environment and has numerous virulence factors that allow for the adherence, colonization, and avoidance of host complement responses. Gonococcal LOS is one of the important surface structures involved in pathogenesis. Due to its accessibility, gonococcal LOS has been proposed to be a vaccine candidate. In particular, the LOS epitope was recognized by MAb 2C7 (44). The MAb 6E4, used in this study, was previously generated by immunizing mice with NTHi LOS (45, 46), and the epitope recognized by 6E4 is KDO (29). KDO is a bacterium-specific carbohydrate that is common to the inner core of almost all Gram-negative bacteria, in which it plays an essential role linking lipid A to the oligo- or polysaccharide portion of LOS/lipopolysaccharide. Using the 6E4 MAb, we have made the unexpected discovery that the KDO epitope is also present on the LOS terminus in *N. gonorrhoeae*. Although the inner core KDO cannot be recognized by 6E4, we show that 6E4 detects a population of LOS molecules that have terminal KDO on LOS in the place where Neu5Ac is normally located. *N. gonorrhoeae* contains one LOS sialyltransferase, Lst, which is known to transfer Neu5Ac from CMP-Neu5Ac to the terminal end of LOS. However, here we have demonstrated that Lst can also use endogenous CMP-KDO as a substrate to transfer KDO to the LOS terminus in the absence of CMP-Neu5Ac.

The expression of KDO as a terminal modification of LOS has been reported in NTHi (29) and is dependent on the sialyltransferase LsgB. The expression of KDO as a terminal modification of LOS in the pathogenic *Neisseria* species is unexpected due to a study that reported that Lst is localized on the outer membrane (41). The earlier study used two experimental approaches to investigate localization, Western blot analysis of subcellular fractions of *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ζ 3 with anti-Lst antiserum and binding of anti-Lst antiserum to whole-cell bacteria in an ELISA. In the cell fractionation and whole-cell binding assay studies, no control proteins (i.e., cytoplasmic, inner membrane, periplasmic, and outer membrane) were used to verify the identity or purity of fractionated material. Similarly, no control proteins were used to establish the status of the whole cells (i.e., intact or lysed) in ELISA. Thus, the conclusion that Lst in the pathogenic *Neisseria* is an outer membrane protein is not supported by strong evidence. Our study presents several lines of evidence that KDO is transferred to LOS by Lst in the cytoplasm. This is consistent with sialyltransferase localization in other Gram-negative pathogens: NTHi (29), *Helicobacter acinonychis*, and *Helicobacter pylori* (47). *N. gonorrhoeae* requires exogenous CMP-Neu5Ac to sialyate LOS and can take up CMP-Neu5Ac from growth media, suggesting a novel CMP-Neu5Ac transport system that delivers CMP-Neu5Ac to the cytoplasm, as shown in Fig. 5. Nucleotide sugar transporters have not been reported in bacterial systems but present eukaryotes to transport nucleotide sugars to the lumen of organelles that are the site of oligosaccharide biosynthesis (48).

Our studies with *N. meningitidis* MC58 ζ 3, which endogenously synthesizes CMP-Neu5Ac, indicate that Neu5Ac is the preferred substrate for Lst. When both CMP-Neu5Ac and CMP-KDO are present in wild-type MC58 ζ 3, only trace amounts of KDO

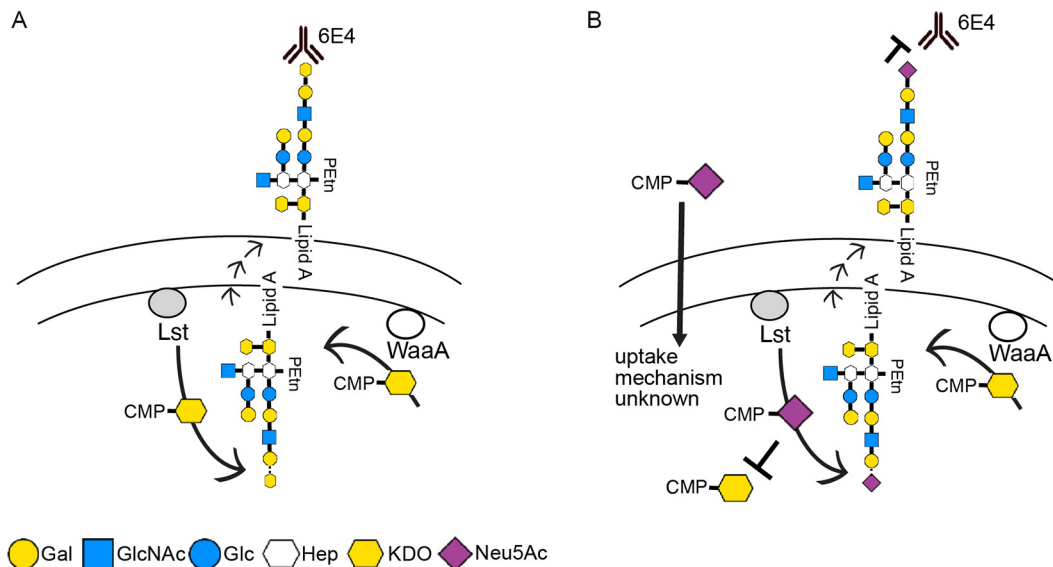


FIG 5 Schematic diagram of proposed biosynthesis pathway of terminal KDO on LOS in *N. gonorrhoeae*. (A) Inside cells, CMP-KDO is synthesized by WaaA and transferred to the terminal glycan of LOS by Lst in the absence of CMP-Neu5Ac. (B) In the presence of CMP-Neu5Ac, CMP-Neu5Ac can be taken up in the intracellular environment by an unknown mechanism and competes with CMP-KDO for addition to LOS by Lst inside cells.

are detectable on LOS, whereas when CMP-Neu5Ac biosynthesis is knocked out (SiaB mutant), KDO is readily detected (Fig. 1B and 2C). In the case of *N. meningitidis* group Y strain, 406Y (NRCC 4030), which expresses L3 immunotype LOS (like MC58 ζ 3), no KDO has been observed linked to the terminal galactose of LOS in nuclear magnetic resonance (NMR) structural analysis (49). Moreover, no terminal KDO was observed in previous mass spectrometry analyses of the *N. gonorrhoeae* strain 1291 LOS structure (17). These studies are consistent with the KDO-terminated LOS representing a very small proportion of all LOS molecules present in the outer membrane. This is consistent with our observation with *N. meningitidis* strain MC58 ζ 3 described above, that CMP-KDO is not the preferred Lst substrate, and suggests that the low level of terminal KDO-LOS is refractory to both NMR and mass spectrometry approaches. Notwithstanding this limitation, the case that KDO is present in the same position as Neu5Ac in LOS of these species is supported by strong evidence, such as (i) the biophysical studies that defined MAb 6E4's epitope as KDO (29), (ii) the Lst dependence of KDO expression on LOS (Fig. 1B and 2A to D), (iii) the requirement for the terminal galactose of LOS for addition of KDO, as evidenced by loss of KDO in a mutant (LgtB) that lacks terminal galactose (Fig. 1B), and (iv) the suppression of KDO addition to LOS by the presence of endogenous CMP-Neu5Ac (*N. meningitidis*, wild type versus SiaB) (Fig. 2C) or exogenous CMP-Neu5Ac (*N. gonorrhoeae*) (Fig. 2D).

In this study, we show that KDO can be transferred to the terminal of LOS by Lst in the absence of Neu5Ac, and the majority of *N. gonorrhoeae* clinical isolates that we surveyed express KDO on their cell surface. This incorporation of KDO also occurs *in vivo*, with KDO detected on *N. gonorrhoeae* colonizing cervical epithelial cells in a cervical swab from a patient with a documented gonococcal infection. The host CMP-Neu5Ac level was not sufficient to suppress KDO incorporation. Significantly, our data showed that there is sufficient KDO-terminated LOS present on the bacterial surface such that MAb 6E4 can bind and kill *N. gonorrhoeae* in an opsonophagocytic killing assay, suggesting that terminal KDO on LOS is a vaccine candidate.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *N. gonorrhoeae* strain 1291, 1291*lst*, MS11, MS11*lst*, F62, and F62*lst* (50) were grown on GC agar (Oxoid) or GC broth with 1% IsoVitalEx, and *N. meningitidis* strain MC58, MC58*lst* (50), and MC58*siaB* (P. Chopra, J. Fühling, P. Ng, T. Haselhorst, J. Dyason, F. Rose, R.

Thomson, R. Gerardy-Schahn, D. Grice, M. Jennings, A. Münster-Kühnel, M. von Itzstein, submitted) were grown on 1% brain-heart infusion (BHI) agar and 10% Levinthal's medium base at 37°C with 5% CO₂. Twenty clinical isolates (35) of *N. gonorrhoeae* from disseminated or mucosal gonorrhoeae infection were also studied. In selected experiments, CMP-Neu5Ac at various concentrations was added to the medium as indicated.

LOS purification. Cells were grown on BHI or GC solid medium. Bacteria were collected by scraping and were suspended in a solution of 60 mM Tris, 10 mM EDTA, and 2% (wt/vol) sodium dodecyl sulfate, pH 6.8. Proteinase K was added to a final concentration of 50 μg/ml, and samples were incubated overnight at 37°C. Following three ethanol precipitations, samples were treated with DNase I and RNase A. Samples were then phenol extracted, ethanol precipitated three more times, and centrifuged at 120,000 × *g* for 75 min. The pellets were resuspended in water, frozen, and then lyophilized.

Antibodies. Monoclonal antibody (MAB) 6E4 is an IgG3 murine monoclonal antibody that recognizes a terminal KDO epitope (46). Polyclonal antisera, α-Lst, were produced by inoculating mice with purified recombinant Lst protein with Freund's adjuvant. The *lst* gene was amplified from MC58 using primers LstNcoI_For (5'-CTGCATCGTAGCCATGGGCTTGAAAAGGCTTG-3') and LstNdeI_Rev (5'-GCACTCGAGCATATGTCAATTAGTGGTATGGTGGTATGATTTTTATCGTCAAATGTCAAATCGGG-3'). The PCR products were cloned into vector pET-15b (Novagen), and the resulting plasmids were transformed into the *Escherichia coli* BL21 Star (DE3)pLysS host strain (Novagen) for overexpression. The expression and purification of Lst are the same as previously described for other neisserial LOS glycotransferases (51, 52).

Whole-cell ELISA. Bacteria were grown on BHI or GC plates for 16 h. Cells were harvested and resuspended in PBS at an optical density at 600 nm of 0.20. Microtiter plate wells were filled with 50 μl of the bacterial suspension and dried at room temperature overnight in the laminar flow cabinet. After the bacteria in the dried wells were heat killed for 1 h at 56°C, the wells were washed and ELISA was performed with MAb 6E4 at a dilution of 1:64. Secondary antibody (polyclonal anti-mouse Ig HRP; P044701; Dako) was used at a dilution of 1:2,000. The substrate TMB (3,3',5,5-tetramethylbenzidine) solution (ThermoFisher Scientific) was used per the manufacturer's instructions. Equal amounts of 1 N hydrochloric acid were added to stop the reaction. Absorbance was read in a Tecan model Infinite 200 Pro plate reader at 450 nm.

Fluorescent labeling of clinical samples. Paraformaldehyde-fixed archival clinical specimens from cervical swabs obtained from patients presenting with gonorrhea were dried onto glass microscope slides.

These samples were incubated simultaneously with MABs 6E4 (IgG) and 6B4 (IgM). Fluorescent label was provided by incubation with goat anti-mouse IgG3/FITC to label MAB 6E4 plus goat anti-mouse IgM/Texas red for MAB 6B4 (both conjugates from Jackson ImmunoResearch). The samples were counterstained with Draq5 (Cell Signaling Technologies) to label cell nuclei and viewed by confocal microscopy on a Leica STED system in the Central Microscopy Core Facility at the University of Iowa.

Human polymorphonuclear leukocyte (PMN) and serum collection. Whole blood from healthy volunteers was collected by venipuncture with informed consent and the approval of the Griffith University Human Ethics Committee (HREC 2012/798). Blood was collected in K3 EDTA or Vacuette Z serum separator tubes (Greiner Bio-One) for PMN or serum preparation, respectively. PMNs were isolated using Polymorphprep (Axis-Shield) per the manufacturer's instructions. For serum isolation, blood was allowed to clot for 15 min at room temperature and then centrifuged for 10 min at 2,000 × *g*.

Oposonophagocytic killing and serum bactericidal assays. A modified version of the oposonophagocytic assay (53) was used, where *N. gonorrhoeae* 1291 (~1 × 10³ CFU) was incubated in serial dilutions of MAB 6E4 (0 to 10 μg) for 15 min at 37°C. PMNs (~1 × 10⁵ cells) and 10% normal human serum as a complement source (preabsorbed with *N. gonorrhoeae* [54]) were then added and incubated at 37°C for 90 min. Serum bactericidal assays were performed by incubating *N. gonorrhoeae* 1291 (~1 × 10³ CFU), MAB 6E4 (0 to 10 μg/ml), and 10% preabsorbed normal human serum for 30 min, as described previously (54). For both assays, gonococcal survival was determined after plating of serial dilutions on GC agar, and survival was calculated as a percentage relative to the no-antibody control. Statistical significance was calculated using one-way analysis of variance (ANOVA) and Student's *t* test.

ACKNOWLEDGMENTS

We are supported by Australian National Health and Medical Research Council (NHMRC) program grant 1071659 and principle research fellowship 1138466 to M.P.J., Ideas grant 2001210 to F.E.J., Career Development Fellowship 1045235 to K.L.S., project grant 1099278 to K.L.S. and C.J.D., and Queensland Government Women's Academic Fund to F.E.-C.J. This work was also supported by NIAID grant AI108255 and NIAID AI024616 from the U.S. Public Health Service.

REFERENCES

- Farley TA, Cohen DA, Elkins W. 2003. Asymptomatic sexually transmitted diseases: the case for screening. *Prev Med* 36:502–509. [https://doi.org/10.1016/s0091-7435\(02\)00058-0](https://doi.org/10.1016/s0091-7435(02)00058-0).
- McCormack WM, Stumacher RJ, Johnson K, Donner A. 1977. Clinical spectrum of gonococcal infection in women. *Lancet* 1:1182–1185. [https://doi.org/10.1016/s0140-6736\(77\)92720-9](https://doi.org/10.1016/s0140-6736(77)92720-9).
- Eschenbach DA. 1984. Acute pelvic inflammatory disease. *Urol Clin North Am* 11:65–81.
- Woods CR. 2005. Gonococcal infections in neonates and young children. *Semin Pediatr Infect Dis* 16:258–270. <https://doi.org/10.1053/j.spid.2005.06.006>.
- Chen A, Boulton IC, Pongoski J, Cochrane A, Gray-Owen SD. 2003. Induction

- of HIV-1 long terminal repeat-mediated transcription by *Neisseria gonorrhoeae*. *AIDS* 17:625–628. <https://doi.org/10.1097/00002030-200303070-00019>.
6. WHO. 2012. Global incidence and prevalence of selected curable sexually transmitted infections—2008. World Health Organization, Geneva, Switzerland.
 7. WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. World Health Organization, Geneva, Switzerland. <http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/>. Accessed 8 April 2020.
 8. CDC. 2013. Antibiotic resistance threats in the United States. <http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf>. Accessed 8 April 2020.
 9. Eyre DW, Sanderson ND, Lord E, Regisford-Reimmer N, Chau K, Barker L, Morgan M, Newnham R, Golparian D, Unemo M, Crook DW, Peto TE, Hughes G, Cole MJ, Fifer H, Edwards A, Andersson MI. 2018. Gonorrhoea treatment failure caused by a *Neisseria gonorrhoeae* strain with combined ceftriaxone and high-level azithromycin resistance, England, February 2018. *Euro Surveill* 23:1800323. <https://doi.org/10.2807/1560-7917.ES.2018.23.27.1800323>.
 10. Unemo M, Del Rio C, Shafer WM. 2016. Antimicrobial resistance expressed by *Neisseria gonorrhoeae*: a major global public health problem in the 21st century. *Microbiol Spectr* 4:10.1128/microbiolspec.E110-0009-2015. <https://doi.org/10.1128/microbiolspec.E110-0009-2015>.
 11. Unemo M, Golparian D, Eyre DW. 2019. Antimicrobial resistance in *Neisseria gonorrhoeae* and treatment of gonorrhoea. *Methods Mol Biol* 1997:37–58. https://doi.org/10.1007/978-1-4939-9496-0_3.
 12. Whiley DM, Jennison A, Pearson J, Lahra MM. 2018. Genetic characterization of *Neisseria gonorrhoeae* resistant to both ceftriaxone and azithromycin. *Lancet Infect Dis* 18:717–718. [https://doi.org/10.1016/S1473-3099\(18\)30340-2](https://doi.org/10.1016/S1473-3099(18)30340-2).
 13. Boslego JW, Tramont EC, Chung RC, McChesney DG, Ciak J, Sadoff JC, Piziak MV, Brown JD, Brinton CC, Jr, Wood SW. 1991. Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine* 9:154–162. [https://doi.org/10.1016/0264-410X\(91\)90147-X](https://doi.org/10.1016/0264-410X(91)90147-X).
 14. Eyre JWH, Stewart BH. 1909. The treatment of gonococcus infections by vaccines. *Lancet* 174:76–81. [https://doi.org/10.1016/S0140-6736\(01\)32510-2](https://doi.org/10.1016/S0140-6736(01)32510-2).
 15. Greenberg L, Diena BB, Ashton FA, Wallace R, Kenny CP, Znamirovski R, Ferrari H, Atkinson J. 1974. Gonococcal vaccine studies in Inuvik. *Can J Public Health* 65:29–33.
 16. Preston A, Mandrell RE, Gibson BW, Apicella MA. 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol* 22:139–180. <https://doi.org/10.3109/10408419609106458>.
 17. Harvey HA, Porat N, Campbell CA, Jennings M, Gibson BW, Phillips NJ, Apicella MA, Blake MS. 2000. Gonococcal lipooligosaccharide is a ligand for the asialoglycoprotein receptor on human sperm. *Mol Microbiol* 36:1059–1070. <https://doi.org/10.1046/j.1365-2958.2000.01938.x>.
 18. Song W, Ma L, Chen R, Stein DC. 2000. Role of lipooligosaccharide in Opaindependent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J Exp Med* 191:949–960. <https://doi.org/10.1084/jem.191.6.949>.
 19. Edwards JL, Apicella MA. 2002. The role of lipooligosaccharide in *Neisseria gonorrhoeae* pathogenesis of cervical epithelia: lipid A serves as a C3 acceptor molecule. *Cell Microbiol* 4:585–598. <https://doi.org/10.1046/j.1462-5822.2002.00212.x>.
 20. Danaher RJ, Levin JC, Arking D, Burch CL, Sandlin R, Stein DC. 1995. Genetic basis of *Neisseria gonorrhoeae* lipooligosaccharide antigenic variation. *J Bacteriol* 177:7275–7279. <https://doi.org/10.1128/jb.177.24.7275-7279.1995>.
 21. Burch CL, Danaher RJ, Stein DC. 1997. Antigenic variation in *Neisseria gonorrhoeae*: production of multiple lipooligosaccharides. *J Bacteriol* 179:982–986. <https://doi.org/10.1128/jb.179.3.982-986.1997>.
 22. Berrington AW, Tan YC, Srikhanta Y, Kuipers B, van der Ley P, Peak IR, Jennings MP. 2002. Phase variation in meningococcal lipooligosaccharide biosynthesis genes. *FEMS Immunol Med Microbiol* 34:267–275. <https://doi.org/10.1111/j.1574-695X.2002.tb00633.x>.
 23. Apicella MA, Mandrell RE. 1989. Molecular mimicry as a factor in the pathogenesis of human neisserial infections: in vitro and in vivo modification of the lipooligosaccharide of *Neisseria gonorrhoeae* by N-acetylneuraminic acid. *Pediatr Infect Dis J* 8:901–902. <https://doi.org/10.1097/00006454-198912000-00033>.
 24. Mandrell RE, Apicella MA. 1993. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. *Immunobiology* 187:382–402. [https://doi.org/10.1016/S0171-2985\(11\)80352-9](https://doi.org/10.1016/S0171-2985(11)80352-9).
 25. Vogel U, Hammerschmidt S, Frosch M. 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitidis* serogroup B are prerequisites for virulence of meningococci in the infant rat. *Med Microbiol Immunol* 185:81–87. <https://doi.org/10.1007/s004300050018>.
 26. Ram S, Shaughnessy J, de Oliveira RB, Lewis LA, Gulati S, Rice PA. 2017. Gonococcal lipooligosaccharide sialylation: virulence factor and target for novel immunotherapeutics. *Pathog Dis* 75:ftx049. <https://doi.org/10.1093/femspd/ftx049>.
 27. Edwards U, Muller A, Hammerschmidt S, Gerardy-Schahn R, Frosch M. 1994. Molecular analysis of the biosynthesis pathway of the alpha-2,8 polysialic acid capsule by *Neisseria meningitidis* serogroup B. *Mol Microbiol* 14:141–149. <https://doi.org/10.1111/j.1365-2958.1994.tb01274.x>.
 28. Gilbert M, Watson DC, Cunningham AM, Jennings MP, Young NM, Wakarchuk WW. 1996. Cloning of the lipooligosaccharide alpha-2,3-sialyltransferase from the bacterial pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *J Biol Chem* 271:28271–28276. <https://doi.org/10.1074/jbc.271.45.28271>.
 29. Apicella MA, Coffin J, Ketterer M, Post DMB, Day CJ, Jen FE, Jennings MP. 2018. Nontypeable *Haemophilus influenzae* lipooligosaccharide expresses a terminal ketodeoxyoctanoate in vivo, which can be used as a target for bactericidal antibody. *mBio* 9:e01401-18. <https://doi.org/10.1128/mBio.01401-18>.
 30. Gulati S, Schoenhofen IC, Whitfield DM, Cox AD, Li J, St Michael F, Vinogradov EV, Stupak J, Zheng B, Ohnishi M, Unemo M, Lewis LA, Taylor RE, Landig CS, Diaz S, Reed GW, Varki A, Rice PA, Ram S. 2015. Utilizing CMP-sialic acid analogs to unravel *Neisseria gonorrhoeae* lipooligosaccharide-mediated complement resistance and design novel therapeutics. *PLoS Pathog* 11:e1005290. <https://doi.org/10.1371/journal.ppat.1005290>.
 31. Gulati S, Schoenhofen IC, Lindhout-Djukic T, Lewis LA, Moustafa IY, Saha S, Zheng B, Nowak N, Rice PA, Varki A, Ram S. 2020. Efficacy of antigonococcal CMP-nonulosonate therapeutics require cathelicidins. *J Infect Dis* 222:1641–1650. <https://doi.org/10.1093/infdis/jiaa438>.
 32. Fox KL, Cox AD, Gilbert M, Wakarchuk WW, Li J, Makepeace K, Richards JC, Munson ER, Hood DW. 2006. Identification of a bifunctional lipopolysaccharide sialyltransferase in *Haemophilus influenzae*: incorporation of disialic acid. *J Biol Chem* 281:40024–40032. <https://doi.org/10.1074/jbc.M602314200>.
 33. Jones PA, Samuels NM, Phillips NJ, Munson RS, Jr, Bozue JA, Arseneau JA, Nichols WA, Zaleski A, Gibson BW, Apicella MA. 2002. *Haemophilus influenzae* type b strain A2 has multiple sialyltransferases involved in lipooligosaccharide sialylation. *J Biol Chem* 277:14598–14611. <https://doi.org/10.1074/jbc.M110986200>.
 34. Wakarchuk WW, Watson D, St Michael F, Li J, Wu Y, Brisson JR, Young NM, Gilbert M. 2001. Dependence of the bi-functional nature of a sialyltransferase from *Neisseria meningitidis* on a single amino acid substitution. *J Biol Chem* 276:12785–12790. <https://doi.org/10.1074/jbc.M011293200>.
 35. Power PM, Ku SC, Rutter K, Warren MJ, Limnios EA, Tapsall JW, Jennings MP. 2007. The phase-variable allele of the pilus glycosylation gene *pgIIA* is not strongly associated with strains of *Neisseria gonorrhoeae* isolated from patients with disseminated gonococcal infection. *Infect Immun* 75:3202–3204. <https://doi.org/10.1128/IAI.01501-06>.
 36. Matthews MM, McArthur JB, Li Y, Yu H, Chen X, Fisher AJ. 2020. Catalytic cycle of *Neisseria meningitidis* CMP-Sialic acid synthetase illustrated by high-resolution protein crystallography. *Biochemistry* 59:3157–3168. <https://doi.org/10.1021/acs.biochem.9b00517>.
 37. Mandrell RE, Lesse AJ, Sugai JV, Shero M, Griffiss JM, Cole JA, Parsons NJ, Smith H, Morse SA, Apicella MA. 1990. In vitro and in vivo modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. *J Exp Med* 171:1649–1664. <https://doi.org/10.1084/jem.171.5.1649>.
 38. Piek S, Kahler CM. 2012. A comparison of the endotoxin biosynthesis and protein oxidation pathways in the biogenesis of the outer membrane of *Escherichia coli* and *Neisseria meningitidis*. *Front Cell Infect Microbiol* 2:162. <https://doi.org/10.3389/fcimb.2012.00162>.
 39. Wakarchuk WW, Cunningham A, Watson DC, Young NM. 1998. Role of paired basic residues in the expression of active recombinant galactosyltransferases from the bacterial pathogen *Neisseria meningitidis*. *Protein Eng* 11:295–302. <https://doi.org/10.1093/protein/11.4.295>.
 40. Hug I, Feldman MF. 2011. Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* 21:138–151. <https://doi.org/10.1093/glycob/cwq148>.
 41. Shell DM, Chiles L, Judd RC, Seal S, Rest RF. 2002. The *Neisseria* lipooligosaccharide-specific alpha-2,3-sialyltransferase is a surface-exposed outer membrane protein. *Infect Immun* 70:3744–3751. <https://doi.org/10.1128/iai.70.7.3744-3751.2002>.

42. Sugai T, Lin CH, Shen GJ, Wong CH. 1995. CMP-KDO synthetase: overproduction and application to the synthesis of CMP-KDO and analogs. *Bioorg Med Chem* 3:313–320. [https://doi.org/10.1016/0968-0896\(95\)00023-a](https://doi.org/10.1016/0968-0896(95)00023-a).
43. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* 8:785–786. <https://doi.org/10.1038/nmeth.1701>.
44. Gulati S, Pennington MW, Czerwinski A, Carter D, Zheng B, Nowak NA, DeOliveira RB, Shaughnessy J, Reed GW, Ram S, Rice PA. 2019. Preclinical efficacy of a lipooligosaccharide peptide mimic candidate gonococcal vaccine. *mBio* 10:e02552-19. <https://doi.org/10.1128/mBio.02552-19>.
45. Abu Kwaik Y, McLaughlin RE, Apicella MA, Spinola SM. 1991. Analysis of Haemophilus influenzae type b lipooligosaccharide-synthesis genes that assemble or expose a 2-keto-3-deoxyoctulosonic acid epitope. *Mol Microbiol* 5:2475–2480. <https://doi.org/10.1111/j.1365-2958.1991.tb02092.x>.
46. Spinola SM, Kwaik YA, Lesse AJ, Campagnari AA, Apicella MA. 1990. Cloning and expression in Escherichia coli of a Haemophilus influenzae type b lipooligosaccharide synthesis gene(s) that encodes a 2-keto-3-deoxyoctulosonic acid epitope. *Infect Immun* 58:1558–1564. <https://doi.org/10.1128/IAI.58.6.1558-1564.1990>.
47. Schur MJ, Lameignere E, Strynadka NC, Wakarchuk WW. 2012. Characterization of alpha2,3- and alpha2,6-sialyltransferases from Helicobacter acinonychis. *Glycobiology* 22:997–1006. <https://doi.org/10.1093/glycob/cws071>.
48. Hadley B, Maggioni A, Ashikov A, Day CJ, Haselhorst T, Tiralongo J. 2014. Structure and function of nucleotide sugar transporters: current progress. *Comput Struct Biotechnol J* 10:23–32. <https://doi.org/10.1016/j.csbj.2014.05.003>.
49. Wakarchuk W, Martin A, Jennings MP, Moxon ER, Richards JC. 1996. Functional relationships of the genetic locus encoding the glycosyltransferase enzymes involved in expression of the lacto-N-neotetraose terminal lipopolysaccharide structure in Neisseria meningitidis. *J Biol Chem* 271:19166–19173. <https://doi.org/10.1074/jbc.271.32.19166>.
50. Mubaiwa TD, Hartley-Tassell LE, Semchenko EA, Jen FE, Srikhanta YN, Day CJ, Jennings MP, Seib KL. 2017. The glycointeractome of serogroup B Neisseria meningitidis strain MC58. *Sci Rep* 7:5693. <https://doi.org/10.1038/s41598-017-05894-w>.
51. Simerska P, Christie MP, Goodwin D, Jen FE, Jennings MP, Toth I. 2013. α -1,4-Galactosyltransferase-catalyzed glycosylation of sugar and lipid modified Leu-enkephalins. *J Mol Catal B Enzym* 97:196–202. <https://doi.org/10.1016/j.molcatb.2013.08.018>.
52. Christie MP, Simerska P, Jen FE, Jennings MP, Toth I. 2013. Liposomes for improved enzymatic glycosylation of lipid-modified lactose enkephalins. *Chempluschem* 78:793–796. <https://doi.org/10.1002/cplu.201300115>.
53. Winter LE, Barenkamp SJ. 2006. Antibodies specific for the high-molecular-weight adhesion proteins of nontypeable Haemophilus influenzae are opsonophagocytic for both homologous and heterologous strains. *Clin Vaccine Immunol* 13:1333–1342. <https://doi.org/10.1128/CVI.00221-06>.
54. Semchenko EA, Day CJ, Seib KL. 2017. MetQ of Neisseria gonorrhoeae is a surface-expressed antigen that elicits bactericidal and functional blocking antibodies. *Infect Immun* 85:e00898-16. <https://doi.org/10.1128/IAI.00898-16>.