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Identification of the Gonococcal *glmU* Gene Encoding the Enzyme *N*-Acetylglucosamine 1-Phosphate Uridyltransferase Involved in the Synthesis of UDP-GlcNAc

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In searching for the gonococcal sialyltransferase gene(s), we cloned a 3.8-kb DNA fragment from gonococcus strain MS11 that hybridized with the oligonucleotide JU07, which was derived from the conserved C terminus of the sialyl motif present in mammalian sialyltransferases. Sequencing of the fragment revealed four putative open reading frames (ORFs), one of which (ORF-1) contained a partial sialyl motif including the amino acid sequence VGSKT, which is highly conserved among sialyltransferases. The gene was flanked by two inverted repeats containing the neisserial DNA uptake sequence and was preceded by a putative σ54 promoter. Database searches, however, revealed a high degree of homology between ORF-1 and the *N*-acetylglucosamine 1-phosphate uridyltransferase (GlmU) of *Escherichia coli* and *Bacillus subtilis* and not with any known sialyltransferase. This homology was further established by the successful complementation of an *orf-1* mutation by the *E. coli glmU* gene. Enzyme assays demonstrated that ORF-1 did not possess sialyltransferase activity but mimicked GlmU function catalyzing the conversion of *N*-acetylglucosamine 1-phosphate into UDP-*N*-acetylglucosamine, which is a key metabolite in the syntheses of lipopolysaccharide, peptidoglycan, and sialic acids.

The lipopolysaccharide (LPS) of the pathogenic *Neisseria* species is unique in that it can undergo external modification through the addition of sialic acids (for a review, see reference 42). This process involves the covalent transfer of sialic acid from the external substrate cytidine 5′-monophospho *N*-acetylneuraminic acid (CMP-NANA) towards terminal galactose moieties on the LPS. The reaction is catalyzed by a membrane-bound bacterial sialyltransferase (26). The substrate CMP-NANA is present in human secretions and biological fluids, and LPS sialylation has been demonstrated to occur in vivo during the natural infection (1). The LPS sialylation event seems to have great impact on the virulence of the bacterium. Sialylated gonococci are resistant against killing by antibodies and complement (9, 34, 41, 47) and are poorly ingested by professional phagocytes (16, 37). Furthermore, LPS sialylation may reduce the immune response towards the bacterium because of the molecular mimicry of host sialic acids and a masking of bacterial surface antigens (25). The ability of the bacterium to change its LPS structure and switch to a nonsialylated phenotype lacking the acceptor sites for sialic acid provides the bacterium with a flexibility to optimally adapt its surface composition to the changing microenvironment in the host. A nonsialylated bacterial phenotype may be particularly useful at those stages in the infection where intimate bacterium-host cell contact and bacterial entry into host cells are advantageous (41).

Despite the apparently important function of bacterial LPS sialyltransferase in the pathogenesis of neisserial disease, our knowledge about this sialyltransferase is still scanty. The enzyme appears to be surface exposed and conserved among meningococcal and gonococcal strains (26). In *Neisseria meningitidis* the LPS sialyltransferase catalyzes the formation of an α2,3 linkage between the sialic acid molecule and terminal galactose residues on the LPS (27, 49). These characteristics clearly distinguish the enzyme from the α2,8-polysialyltransferase that is involved in the synthesis of the polysialic capsule in group B and C meningococci (8, 10). The genes for these enzymes are absent in gonococci, and these enzymes are not at the bacterial cell surface. In the present study we aimed to identify the gene(s) encoding the gonococcal α2,3-LPS sialyltransferase, taking advantage of a highly conserved DNA sequence that is present in mammalian sialyltransferases. This sequence, encoding the amino acids DVGSKT, is located at the C-terminal part of the so-called sialyl motif that is likely to be involved in the recognition of the substrate CMP-NANA (6). This approach resulted in the identification of a gene that contains a putative CMP-NANA binding site but does not possess sialyltransferase activity. In contrast, detailed genetic, biochemical, and functional analyses identified the gene as the gonococcal *glmU* homolog encoding the enzyme *N*-acetylglucosamine 1-phosphate (GlcNAc 1-P) uridyltransferase (also called uridine diphosphoglucosamine pyrophosphorylase; EC 1.7.7.23). This enzyme catalyzes the synthesis of UDP-GlcNAc, which is a key precursor in the biosynthesis of LPS, peptidoglycan, and sialic acids.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Neisseria gonorrhoeae* MS11-A42 and MS11-F3 are highly piliated variants of the progenitor variant MS11A (33). The *Escherichia coli* strain DH5α [F^- ΦF80d*lacZ*ΔM15 Δ(*lacZYAargF*)U169 *deoR recA1 endA1 phoA hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*; Gibco] was used as a host strain for cloning with the Hermes and Bluescript SK^+ plasmids. *E. coli* GC7 [MDU *supE44* (*recA-srT*)$_{306}$ *hsdR*$^-$*M*$^+$ *thi-1 lac*] was used for Southern hybridizations. Gonococcal strains were routinely grown (37°C, 5% CO_2) on a Gc agar base supplemented with 1% vitamins. Gonococcal recombinants were grown on plates containing either 7 μg of erythromycin per ml (for MS11 ptetM::*glmU_Ec* and MS11 ptetM::*orf-1*) or 7 μg of erythromycin per ml, 10 μg of chloramphenicol per ml, and 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (for MS11 ptetM::*glmU_Ec*/Δ*orf-1cat* and MS11 ptetM::*orf-1*/Δ*orf-1cat*). *E. coli* DH5α and its derivatives were grown (37°C) on Luria-Bertani plates supplemented with erythromycin (250 μg/ml) or ampicillin (100 μg/ml) to main-

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
M13 reverse	5′-AACAGCTATGACCATG-3′
JU05[a]	5′-TGCCGYCGYTGCGCVGTHGTHGG-3′
JU07[a]	5′-GTNGTTTTGCTRCCDACRTC-3′
JU08	5′-GCGGGATCCACAGGAAAACGCTATGCCGC-3′
JU09	5′-GCGACATCTAAGCTTGGTTTGCCGTCCTTATCG-3′
JU10	5′-GCGGGATCCGTCAGGACGCGTATGTTGAATAATGC-3′
JU11	5′-GCGAAGCTTCATTGTCGGTCAACCTGTATGCCC-3′

[a] Presented in the standard ambiguous assignment code for nucleotides.

tain the Hermes 11 and the pBluescript SK^+ vectors, respectively. Chloramphenicol (100 μg/ml) and/or IPTG (0.1 mM) was added when appropriate. For measurements of bacterial enzyme activities, MS11 and *E. coli* strains were grown on a rotatory shaker (150 rpm) in 40 ml of Proteose Peptone medium enriched with 1% vitamins, erythromycin, and IPTG for a period of 2 h (37°C).

Cloning of the *glmU* gene of *N. gonorrhoeae* and *E. coli*. Southern analysis to identify JU07 (Table 1)-hybridizing DNA fragments was performed as described by Maniatis et al. (28). Nylon blots were incubated overnight at 42°C with digoxigenin-labeled JU07 and washed twice in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 45°C prior to development. A cDNA clone carrying the porcine Galβ1,3GalNAc α2,3-sialyltransferase gene (12) was generously provided by J. C. Paulson. The sialyl motif of this gene, which served as a positive control in the Southern analysis, was PCR amplified with the oligonucleotides JU05 and JU07 (Table 1) and cloned into pBluescript SK^+. The JU07-hybridizing gonococcal *Eco*RI-*Bcl*I-DNA fragment was purified from a 0.8% TAE (40 mM Tris-acetate, pH 7.5, plus 1 mM EDTA)-agarose gel by electroelution and cloned into the pBluescript vector. Transformants were selected on ampicillin plates and checked by Southern hybridization. The oligobinding site was mapped by PCR with the M13 reverse primer of pBluescript (Table 1) and JU07. The PCRs were done in a Perkin-Elmer Cetus thermal cycler in a final volume of 100 μl containing 100 pmol of primers, 100 ng of template DNA, and 1 U of VentR polymerase (New England Biolabs), with 25 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min 30 s at 72°C. The *E. coli glmU* gene was PCR amplified (30 cycles of 1 s at 94°C, 5 s at 60°C, and 1 min 30 s at 72°C) from strain DH5α with 50 pmol of the primers JU10 and JU11 (JU10 and JU11, positions 10824 to 10850 and 12322 to 12347 of the *unc* operon [42], respectively) (Table 1), 100 ng of template DNA, and 1 U of VentR polymerase. The presence of *Bam*HI and *Hin*dIII restriction sites in JU10 and JU11, respectively, served to facilitate cloning of the gene into the plasmids. PCR products were purified on a 0.8% TAE-agarose gel and separated from the gel matrix with Geneclean (Bio 101, La Jolla, Calif.). Enzymes and buffers for DNA restriction, ligation, and blunt ending (T4 polymerase) were from Boehringer Mannheim and were used according to the manufacturer's protocols.

DNA sequencing. For sequencing, subclones (300 to 500 bp) of a 2.5-kb *Cla*I-*Eco*RI fragment of pSK::*ST1* containing the JU07 oligobinding site were constructed in pBluescript SK^+ (Stratagene) and sequenced with an Applied Biosystems model 373 automated sequencer. Sequences were analyzed with the programs TFASTA, BESTFIT, and GAP from the Genetics Computer Group software package (7). The isoelectric point was calculated by the CHARGPRO program (PC Gene; IntelliGenetics, Inc.).

Expression of *glmU* in *E. coli*. The expression and shuttle vector pHermes 11, carrying either the *E. coli glmU* gene or gonococcal *orf-1* under the control of an IPTG-inducible promoter, was introduced into *E. coli* DH5α by electroporation of 50 ng of DNA with a Bio-Rad Gene Pulser (capacitance, 25 μF; voltage, 2.5 kV; resistance, 200 Ω). Transformants were selected on Luria-Bertani plates containing erythromycin (250 μg/ml). The expression of the gonococcal gene was analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (21) of cytosolic and membrane fractions of *E. coli* harboring either the plasmid pHermes 11::*orf-1* or the vector alone (pH11) after growth in Luria-Bertani medium in the presence of 0.2 mM IPTG for 30 min. Cells were fractionated by sonication with a Branson Sonifier (settings, 60% duty cycle, position 5, 5 min, and 4°C) followed by centrifugation (15,000 × *g*, 15 min). Proteins were visualized by silver staining (3). The pHermes 11 vector was generously provided by Thomas F. Meyer (Max-Planck-Institut für Biologie, Tübingen, Germany). The construction of the Hermes vectors has been described elsewhere (18, 19).

Construction of gonococcal *glmU* mutants. The MS11 strains with defective chromosomal copies of *orf-1* were constructed in two steps. First, gonococcal *orf-1* or *E. coli glmU* was cloned into the shuttle vector pHermes 11 under the control of an IPTG-inducible promoter and was transformed into gonococcus strain MS11-A42, with erythromycin resistance used as a marker for integration of the cloned genes into the endogenous gonococcal plasmid ptetM25.2. Gonococcal transformation was carried out as described previously (38). In the next step, these strains (MS11 ptetM::*orf-1* and MS11 ptetM::*glmU*) were transformed with plasmid pSK::*ST1*Δ*orf-1cat*. This plasmid was constructed by performing *Bsm*I digestion of pSK::*ST1*, blunt ending the overhanging ends with T4 polymerase, and introducing a blunt-ended *cat* (chloramphenicol acetyltransferase) marker gene under the control of an *opa* promoter (19). Transformants defective in the chromosomal copy of *orf-1* were selected on erythromycin-, chloramphenicol-, and IPTG-containing plates. The mutagenesis was verified by Southern hybridizations with peroxidase-labelled *cat* or *orf-1* as probes. The filters were washed twice in 0.1 SSC at 55°C and developed with the ECL detection system (Amersham).

Assay for GlcNAc 1-P uridyltransferase activity. Cell extracts for the enzyme assays were prepared from 2- to 3-h exponentially grown 40-ml cultures. Bacteria (5×10^8/ml) were then collected by centrifugation (7,5000 × *g*, 30 min, 4°C), resuspended in 400 μl of 50 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication for 5 min on ice (with a Branson Sonifier set at position 4). Enzymatic activity was measured by analysis of the formation of [^{32}P]UDP-GlcNAc from GlcNAc 1-P after addition of 10 μl of bacterial extract (0.5 mg of protein) to 40 μl of prewarmed (37°C) 50 mM Tris-HCl buffer (pH 8.0) containing 4 mM GlcNAc 1-P (Sigma), 3 mM UTP, 8 mM $MgCl_2$, 20 mM sodium fluoride, and 0.2 μM [α-^{32}P]UTP (specific activity, 3,000 Ci/mmol; Amersham). When appropriate, 1.25 mM CMP-NANA was included in the mixture. The enzymatic reaction was linear for up to 30 min but was usually stopped after 1, 3, 6, and 10 min by transferring 10-μl aliquots of the mixture into 5 μl of 0.33 M formic acid containing 5 mM unlabelled UDP-GlcNAc (Sigma). Reaction products were separated on 0.1-mm-thick, 20-cm-long polyethyleneimine cellulose thin-layer chromatography plates (Merck) with a mixture of borate buffer (0.5 M, 70 ml) and ethylene glycol (25 ml) as described by Randerath and Randerath (36). The plates were then exposed to X-ray film for up to several hours. Enzyme assays run without the substrate GlcNAc 1-P and the thin-layer chromatography migration of unlabelled UDP-GlcNAc (Sigma), which can be visualized under UV light, served to identify the reaction products. The enzymatic reaction was also quantitated by liquid scintillation counting of the various spots on the thin-layer chromatography plate.

Sialyltransferase assay. Bacterial sialyltransferase activity was determined in 100 μl of imidazole buffer (25 mM imidazole, pH 6.5, 10 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100) containing 20 μl of stock solution of asialofetuin (Fluka) (20 mg/ml), 1 μl of CMP-[^{14}C]NANA (269 mCi/mmol, 25 mCi/ml; Amersham), and 79 μl of bacterial extract. After 60 min of incubation (37°C), 100 μl of acetone was added and the proteins were pelleted (15,000 × *g*, 15 min), washed once with 100 μl of 50% acetone, and pelleted again. The pellet was dissolved in 50 μl of distilled water, and incorporated radioactivity was counted in a Beckman liquid scintillation counter. Under these conditions, the incorporation of sialic acid into asialofetuin was linear for up to 2 h. Extracts prepared from gonococcal strain MS11 *galE* (38), which lacks the LPS acceptor site for sialic acid, served as a positive control. All bacterial extracts were prepared by sonication for 30 s on ice (with a Branson B15 Sonifier set at position 5 and 60% duty cycle) of bacteria (10^7 CFU/ml) suspended in imidazole buffer. LPS sialylation of gonococcal strain MS11 ptetM::*glmU*_ *Ec*/Δ*orf1-cat* was determined by resuspending 10^6 bacteria in 100 μl of phosphate-buffered saline (PBS) containing 1 μl of 1 μM CMP-[^{14}C]NANA, incubating the mixture for 15 min at 37°C, and separating the bacteria and the label by centrifugation (5,000 × *g*, 5 min). After two additional washes with 100 μl of PBS to remove free CMP-[^{14}C]NANA, the bacterial pellet was resuspended in 100 μl of H_2O and radioactivity was determined in a liquid scintillation counter. In this experiment, the LPS *galE* mutant served as a negative control.

Nucleotide sequence accession number. The sequence reported in this study is available in the EMBL data library under accession no. Z50023.

RESULTS

Cloning and sequence analysis of a gonococcal gene containing a putative sialic acid binding motif. In our search for the gonococcal α2,3-sialyltransferase gene, we designed a degenerate oligonucleotide (JU07) (Table 1) encompassing the C terminus of the sialyl motif, which is highly conserved among mammalian sialyltransferases and supposedly acts as a recognition site for the substrate CMP-NANA (6). Southern blots with digoxigenin-labelled JU07 as a probe demonstrated strong hybridization of JU07 with a single, approximately 4.5-kb DNA *Cla*I-fragment of gonococcus strain MS11-F3 and with the sialyl motif of the Galβ1,3GalNAc α2,3-sialyltransferase gene, which served as a positive control (Fig. 1). Under the same stringent washing conditions, no hybridization was observed with DNA isolated from *E. coli* GC7 that lacks α2,3-sialyltransferase activity (Fig. 1). To further analyze the hybridizing gonococcal DNA fragment, the appropriate band was isolated from

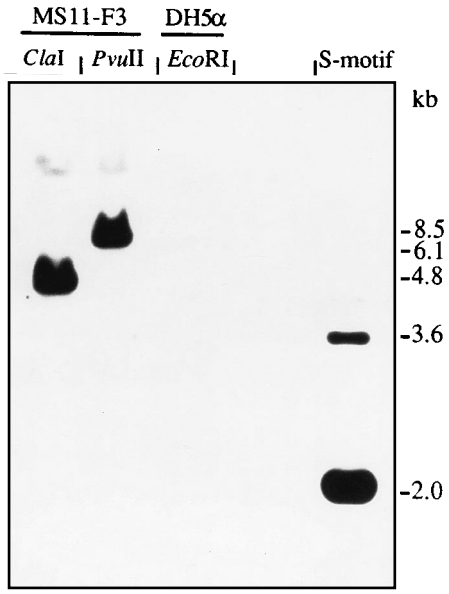

FIG. 1. Southern blot demonstrating the hybridization of oligonucleotide JU07 on *Cla*I- and *Pvu*II-digested chromosomal DNA of gonococcus strain MS11-F3 and on pBluescript carrying the sialyl motif (S-motif) of the porcine Galβ1,3GalNAc α2,3-sialyltransferase gene. No hybridization was observed with *Eco*RI-digested DNA of *E. coli* DH5α which lacks α2,3-sialyltransferase activity.

*Eco*RI- and *Bcl*I-digested gonococcal DNA by electroelution and was ligated into the vector pBluescript SK$^+$ to form pSK::*ST1* (Fig. 2a). Transformation of this plasmid into *E. coli* DH5α and selection for ampicillin resistance resulted in several stable JU07-positive transformants, as was confirmed by Southern hybridization and PCR with the M13 reverse primer of pBluescript SK$^+$ and JU07 (data not shown). For sequence determination, subclones of a 2.5-kb fragment containing the oligonucleotide binding site were constructed in pBluescript and sequenced (Fig. 3). Computer-aided analysis of the sequence revealed four open reading frames (ORFs) (ORF-1 to ORF-4). The largest one (ORF-1) consisted of 1,368 bp (Fig. 3) and was predicted to encode a protein of 456 amino acids with a molecular mass of 48.8 kDa and a pI of 6.9. A putative Shine-Dalgarno site for translation initiation was located 7 bp upstream of the putative ATG start codon, and a potential σ54 promoter with an unusual 11-bp spacing between the −12 and −24 regions was identified 84 bp upstream of the coding region (Fig. 3). Between the putative ribosomal binding site and the promoter and also immediately downstream of ORF-1, two inverted repeats of approximately 30 bp that contained the neisserial DNA uptake sequence (13) were present. ORF-1 also contained a 33-amino-acid polypeptide with 15 identical and 8 conserved amino acid residues common to the mammalian sialyl motif, including the sequence VGSKT, which served as the basis for the oligonucleotide JU07 (Fig. 4). A second incomplete ORF (ORF-2) was transcribed in the same direction away from ORF-1 (Fig. 3). The first 79 amino acids of this putative protein showed 66% identity (73% similarity) at the amino acid level with the product of the *phnA* gene of the alkylphosphonate operon of *E. coli* (5). In the opposite direction, two further ORFs, encoding putative proteins of 158 amino acids (ORF-3) and 187 amino acids (ORF-4) were identified. No significant sequence homologies were found for these ORFs in the databases, and these potential genes were not further analyzed.

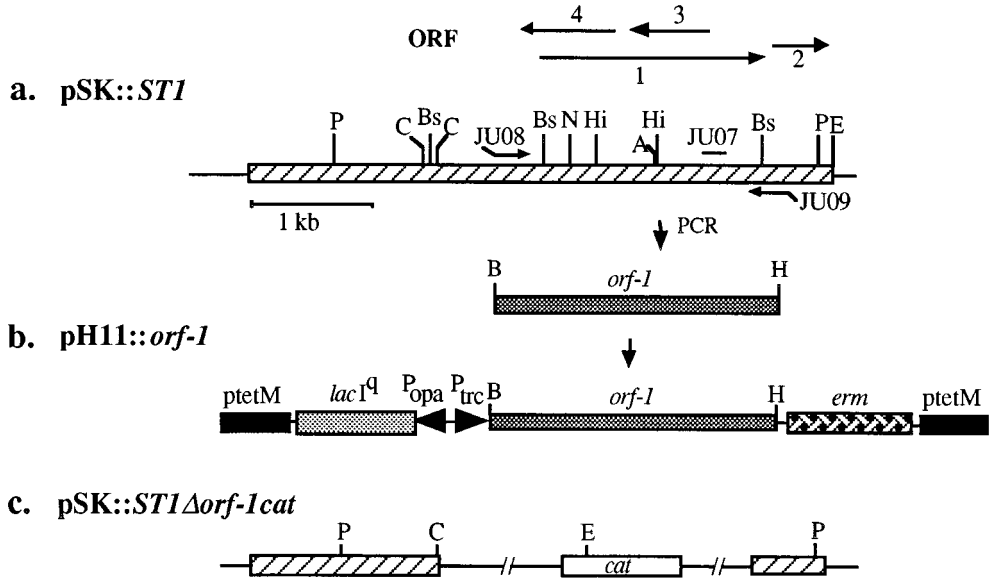

FIG. 2. Schematic representation of plasmid constructs used in this study. (a) Restriction sites of pSK::*ST1* and positions of oligonucleotides used in this study. The sequenced part of pSK::*ST1* contains four ORFs (ORF-1 to ORF-4). JU07 indicates the binding site for this oligonucleotide in ORF-1. JU08 and JU09 are oligonucleotides used to PCR amplify and clone ORF-1 into pBluescript and pHermes 11, generating pSK::*orf-1* and pH11::*orf-1*, respectively. Insertion of a chloramphenicol resistance cartridge into the *Aat*II site in *orf-1* generated the plasmid pSK::*orf-1*Δ*cat*, which was initially used to create an *orf-1* mutant (see text). (b) Map of pH11::*orf-1*. Shown is the position of the IPTG-inducible *orf-1* between two ptetM sequences enabling integration of the inducible element into the endogenously gonococcal ptetM25.2 plasmid via reciprocal recombination. The P_{trc} promoter is repressed by the *lacI*^{q} gene product and is active in the presence of IPTG. P_{opa} is a gonococcal opacity protein promoter which is active in *E. coli* and *N. gonorrhoeae*. *erm* is an erythromycin resistance gene enabling selection for recombination of the cloned genes into the conjugative plasmid ptetM25.2 (19). (c) Restriction map of pSK::*ST1*Δ*orf-1cat*. In this construct the complete DNA region between the *Bsm*I sites of pSK::*ST1* is deleted and replaced by a chloramphenicol resistance cartridge (*cat*). This plasmid was used to mutagenize the chromosomal copy of *orf-1*. Abbreviations for restriction enzymes: P, *Pvu*II; C, *Cla*I; Bs, *Bsm*I; N, *Nco*I; Hi, *Hin*dIII; A, *Aat*II; E, *Eco*RI; B, *Bam*HI; H, *Hin*cII.

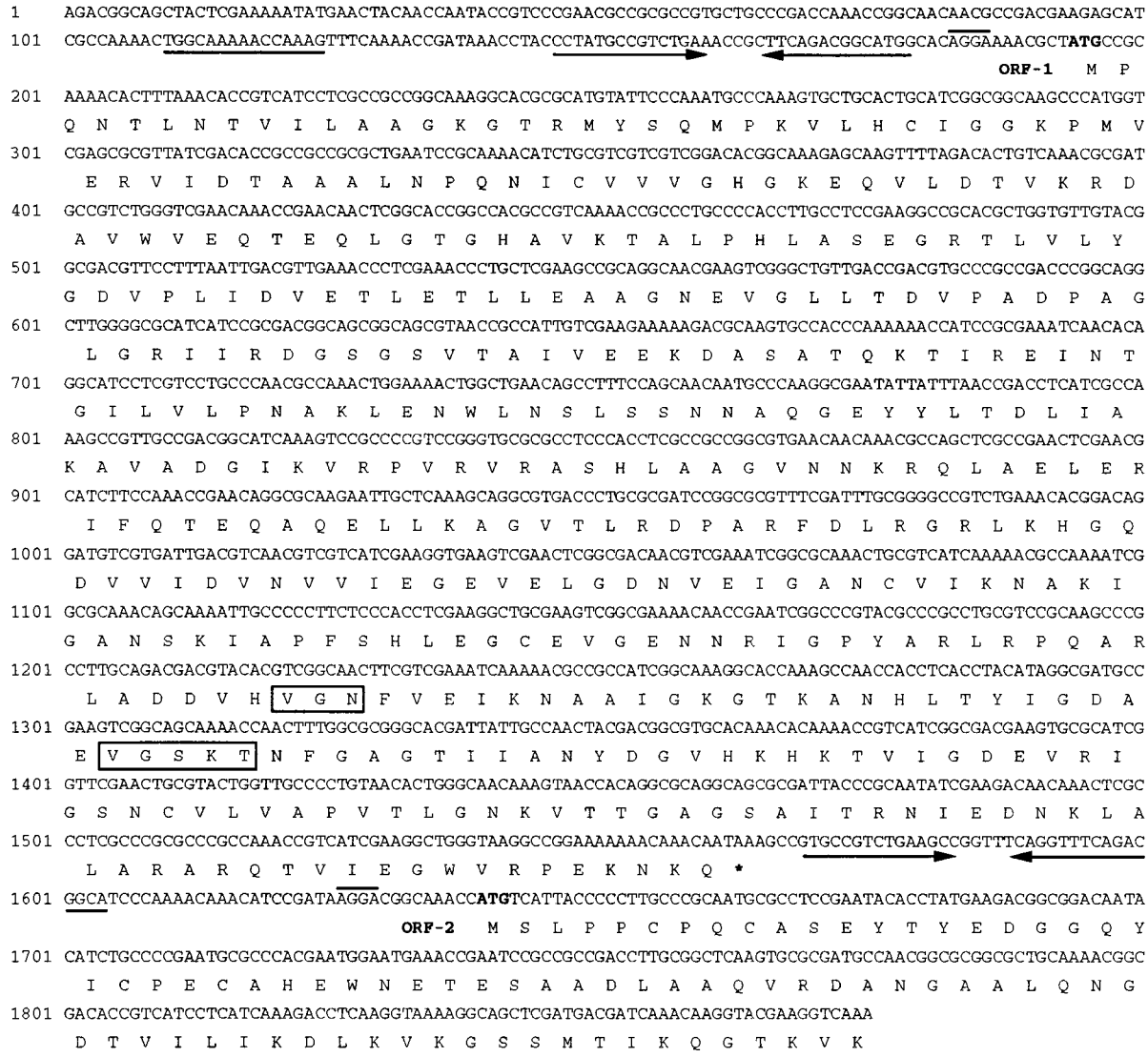

FIG. 3. Oligonucleotide and deduced amino acid sequences of the *orf-1* region. *orf-1* is positioned between nucleotides 194 and 1562. *orf-2* is located downstream, starting at position 1639. The putative start codons are given in boldface letters; Shine-Dalgarno consensus sequences are indicated by lines above the sequence. An asterisk marks the stop codon for *orf-1*. The putative σ54 promoter of *orf-1* is underlined; inverted repeats containing the neisserial DNA uptake sequence are marked by arrows. The N and C termini of the putative sialyl motif in *orf-1* are boxed.

Homology of ORF-1 with bacterial GlmU. Using the program PILEUP (Genetics Computer Group software), we compared the deduced amino acid sequence of ORF-1 with data in the SwissProt data bank, and this comparison revealed 70% similarity (54% identity) with the uridyltransferase (GlmU) of *E. coli* (30) and 59% similarity (41% identity) with the corresponding enzyme of *B. subtilis* (15) (Fig. 5). This homology was found over the entire length of the sequence. Significant homology of ORF-1 with known sialyltransferases was not found, though an incomplete sialyl motif was identified in the GlmU protein of *E. coli* but not in that of *B. subtilis*. All three proteins contained incomplete tandem hexapeptide repeats with the consensus sequence (L/I/V)(G/X)XXXX (Fig. 5), which appears to be a characteristic of bacterial acetyl- and acyltransferases (40). In *E. coli*, the *glmU* gene product has been demonstrated to catalyze both the acetylation of glucosamine 1-phosphate (31) and the subsequent conversion of the formed GlcNAc 1-P into UDP-GlcNAc (31). UDP-GlcNAc is a key

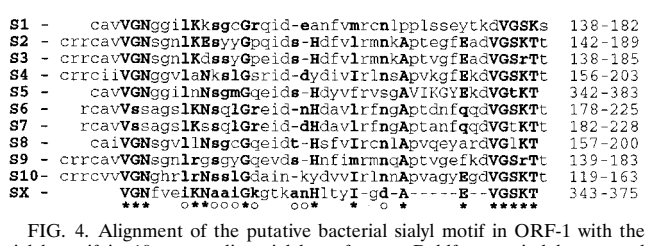

FIG. 4. Alignment of the putative bacterial sialyl motif in ORF-1 with the sialyl motif in 10 mammalian sialyltransferases. Boldface capital letters and asterisks mark well-conserved amino acids (present in at least three of the other sequences). Boldface lowercase letters and open circles indicate equivalent amino acids (present in at least three sialyltransferases). The following residues were defined as equivalent: FYW, MLVI, ATSG, KR, NDEQ (4). Abbreviations: S1, GM3 α2,8-sialyltransferase (39); S2, Galβ1,3GalNAc α2,3-sialyltransferase (12); S3, Galβ1,3GalNBAc α2,3-sialyltransferase (23); S4, Galβ1,3(4) GlcNAc α2,3-sialyltransferase (46); S5, GalNAc α2,6-sialyltransferase (20); S6, Galβ1,4GlcNAc α2,6-sialyltransferase (45); S7, Galβ α2,6-sialyltransferase (14); S8, sialyltransferase of unknown specificity (24); S9, Galβ1,3GalNAc α2,3-sialyltransferase (22); S10, Galβ1,3GalNAc α2,3-sialyltransferase (17); SX, ORF-1 of *N. gonorrhoeae*.

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ORF1_Ng: MPQNTL NTVILA AGKGTR MYSQM PKVLHC IGGKPM VERVID TAAALN PQNICVV VGHGKEQ VLDTVK  (1- 67)
GlmU_Ec: MLNNAM SVVILA AGKGTR MYSDL PKVLHT LAGKAM VQHVID AANELG AAHVHLV YGHGGDL LKQALK
GlmU_Bs: M--DKR FAVVLA AGQGTR MKSKL YKVLHP VCGK-M VEHVVD EALKLS LSKLVTI VGHGAEE VKKQLG

ORF1_Ng: RDAV-W VEQTEQ LGTGHA VKTALP HLAS- EGRTLV LYGDVP LIDVET LETLLE A--AGNE- VGLLTD  (68-129)
GlmU_Ec: DDNLNW VLQAEQ LGTGHA MQQAAP FFADD EDILML -YGDVP LISVET LQRLRD AKPQGG-- IGLLT-
GlmU_Bs: DKSEYR V-QAKQ LGTAHA VKQAQP FLAD- EKGVTV ICGDTP LLTAET MEQMLK EHTQREAK RTILTA

ORF1_Ng: V-PADP AGLGRIIR DGSGSV TAIVEE KDASAT QKTIRE INTGIL VLPNAK LENWL  NSLSSN NAQGE  (130-194)
GlmU_Ec: VKLDDP TGYGRITR -ENGKV TGIVEH KDATDE QRQIQE INTGIL IANGAD MKRWL  ANVTNN NAQGE
GlmU_Bs: V-AEDP TGYGRIIR SENGAV QKIVEH KDASEE ERLVTE INTGTY CFDNEA LFRAI  DQVSND NAQGE

ORF1_Ng: YYLTDL IAKAVA DGIKVRP VRVRASH LAAGVN NK-QLA ELERI- FQTE-Q AQELLK AGVTLR DPARFD  (195-260)
GlmU_Ec: YYITDI IALAYQ EGREIVA VHPQRLS EVEGVN NRLQLS RLERV- YQSE-Q AEKLLL AGVMLR DPARFD
GlmU_Bs: YYLPDV IEILKN EGETVAA YQTGNFQ ETLGVN DRVALS QAEQFM K--ERI NKRHMQ NGVTLI DPMNTY

ORF1_Ng: LRGRLK HGQDVV IDVNVV IEGEVE LGDNVE IGANCV IKNAK IGANSK IAPFSH LEGCE  VGENNR  (261-324)
GlmU_Ec: LRGTLT HGRDVE IDTNVI IEGNVT LGHRVK IGTGCV IKNSV IGDDCE ISPYTV VEDAN  LAAACT
GlmU_Bs: ISPDAV IGSDTV IYPGTV IKGEVQ IGEDTI IGPHTE IMNSA IGSRTV IKQ- V VNHSK  VGNDVN

ORF1_Ng: IGPYAR LRPQAR LADDVH VGNFVE IKNAA IGKGTK ANHLTY IGDAE VGSKTN FGAGTI IANYDGV  (325-389)
GlmU_Ec: IGPFAR LRPGAE LLEGAH VGNFVE MKKAR LGKGSK AGHLTY LGDAE IGDNVN IGAGTI TCNYDGA
GlmU_Bs: IGPFAH IRPDSV IGNEVK IGNFVE IKKTQ FGDRSK ASHLSY VGDAE VGTDVN LGCGSI TVNYDGK

ORF1_Ng: HKHKTV IGDEVR IGSNCV LVAPVT LGNKVT TGAGSA ITRNIE DNKLAL ARARQT VIEGWV RPEKNK  (390-456)
GlmU_Ec: HKFKTI IGDDVF VGSDTQ LVAPVT VGKGAT IAAGTT VTRNVG ENALAI SRVPQT QKEGWR RPVKKK
GlmU_Bs: HKYLTK IEDGAF IGCNSN LVAPVT VGEGAY VAAGST VTEDVP GKALAI ARARQV NKDDYV KNIHKK
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FIG. 5. Alignment of the deduced amino acid sequence for *N. gonorrhoeae* ORF-1 (ORF1_Ng) with the uridyltransferases of *E. coli* (GlmU_Ec) and *B. subtilis* (GlmU_Bs). Amino acids identical to the ORF-1 sequence are indicated by boldface letters. Glycines (G) (or equivalent alanines [A]) at positions 2 and 4 of the incomplete hexapeptide repeat motif are marked with open circles above the sequence; Val (V), Leu (L), or Ile (I) residues that often precede a glycine are marked with asterisks. The repetitive structure of the sequence is particularly apparent in the C-terminal part of the protein.

intermediate in the biosyntheses of peptidoglycan, LPS, and CMP-NANA, the last of which is a precursor in the formation of polysialic acid capsules in *E. coli* and *N. meningitidis* (11, 29, 35).

Expression and enzyme activity of the *orf-1* gene product in *E. coli*. In order to enable functional analysis of the *orf-1* gene product, the gene, including its putative ribosomal binding site and putative terminator sequences, was PCR amplified from pSK::*ST1* with the oligonucleotides JU08 and JU09 (Fig. 2) and cloned into the expression vector pHermes 11 (Fig. 2b). The resulting construct (pH11::*orf-1*) was then introduced into *E. coli* DH5α by transformation, and recombinants were selected for erythromycin resistance. SDS-PAGE analyses of isolated cytosolic and membrane fractions of the transformants (DH5α pH11::*orf-1*) and of the parental strain harboring the vector alone (DH5α pH11) demonstrated the presence of an additional protein with an apparent molecular mass of approximately 50 kDa in DH5α pH11::*orf-1* only (Fig. 6). This size corresponds to the predicted molecular mass of 48.8 kDa for the *orf-1* gene product and strongly suggests that *orf-1* was transcribed in *E. coli*.

To determine the enzymatic activity of the *orf-1* gene product, we prepared crude extracts of the recombinant *E. coli* strains and measured their abilities to convert GlcNAc 1-P into UDP-GlcNAc in the presence of [α-^{32}P]UTP. The labelled products were separated by polyethyleneimine-cellulose thin-layer chromatography and visualized by autoradiography. The reaction was also quantitatively followed by scintillation counting of the formed product. The formation of [^{32}P]UDP-GlcNAc was confirmed by its comigration with unlabelled UDP-GlcNAc, which can be visualized under UV light, and by reactions in the

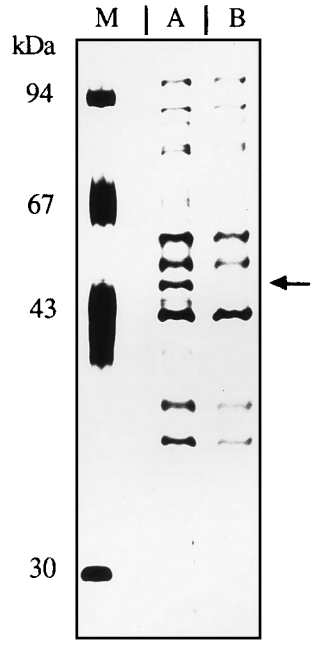

FIG. 6. SDS-PAGE demonstrating the expression of *orf-1* in *E. coli*. Lanes: M, molecular mass markers; A, DH5α harboring the *orf-1* gene on the Hermes 11 vector (DH5α pH11::*orf-1*); B, DH5α carrying the plasmid without insert (DH5α pH11). Both strains were grown in the presence of 0.1 mM IPTG. The induced protein is marked with an arrow.

TABLE 2. GlmU activities of the various wild-type and mutant strains of *E. coli* and *N. gonorrhoeae*[a]

Strain and plasmid	Amt of UDP-GlcNAc formed (nmol min^{-1} mg of $protein^{-1}$)	Amplification (fold)
E. coli DH5α		
pH11	69	1.0
pH11::*orf-1*	110	1.6
pH11::*glmU_Ec*	530	7.7
N. gonorrhoeae MS11		
ptetM	8.4	1.0
ptetM::*orf-1*/Δ*orf-1cat*	44.4	5.3
ptetM::*glmU_Ec*/Δ*orf-1cat*	266.0	31.7
ptetM[b]	4.4	0.6

[a] Strains were exponentially grown in broth in the presence of 0.1 mM IPTG at 37°C for 3 h. Cell extracts were prepared and enzyme activities were determined as described in Materials and Methods. Results are the means of three experiments.
[b] Plus 1.25 mM CMP-NANA.

absence of the substrate GlcNAc 1-P. The results of the experiments are summarized in Table 2. The level of GlmU activity in the recombinant *E. coli* strain expressing the *orf-1* gene product (DH5α pH11::*orf-1*) was only about 1.6-fold higher than that measured for the recipient strain harboring the vector alone (DH5α pH11). In contrast, when the *E. coli glmU* gene was amplified by PCR, cloned into the same vector, and expressed in the same *E. coli* recipient strain (generating DH5α pH11::*glmU_Ec*), a 7.7-fold increase in the level of enzyme activity was measured. These data indicate that the gonococcal ORF-1 may carry GlmU activity but that the *E. coli* homolog is much more active in the *E. coli* background. DH5α pH11::*orf-1* did not possess detectable LPS sialyltransferase activity (data not shown).

Functional complementation of a gonococcal *orf-1* mutant by the *E. coli glmU* gene. The possible functional homology between the gonococcal ORF-1 and *E. coli* GlmU was further established by the construction and complementation of a gonococcal mutant with a defect in *orf-1*. First we attempted to disrupt ORF-1 function by transforming gonococcus strain MS11-A42 with a gonococcal suicide plasmid which carries a chloramphenicol resistance cartridge into the *Aat*II site of the *orf-1* gene so that the promoter was oriented downstream (plasmid pSK::*orf-1*Δ*cat*). However, gonococcal null mutants could not be established by this method. In several attempts a few chloramphenicol-resistant transformants were obtained, but in all these cases allelic replacement of the chromosomal *orf-1* by the defective copy had not occurred and the resistance cassette was located in a gene other than the target gene (data not shown). These data indicate a strong negative selection for the sought mutation and suggest that *orf-1* may have a vital housekeeping function, which is in agreement with its possible function as a GlmU.

To avoid this problem, we then applied the strategy of mutagenesis of *orf-1* in a gonococcal strain carrying either an intact copy of *orf-1* or the *E. coli glmU* gene on the endogenous gonococcal ptetM25.2 plasmid under the control of an IPTG-inducible promoter (Fig. 2b). These genes were introduced onto this plasmid with the pHermes 11 shuttle vector (18). This vector can replicate in *E. coli* but not in *Neisseria* spp. However, because of the presence of ptetM sequences flanking the plasmid insert, the vector can be used to introduce genes onto the endogenous gonococcal ptetM25.2 plasmid via reciprocal recombination. Selection of transformants for erythromycin resistance serves as an indicator of the recombination event (18). This procedure resulted in the generation of two strains, MS11 pH11::*orf-1* and MS11 pH11::*glmU_Ec*, that carry the gonococcal *orf-1* and the *E. coli glmU* gene, respectively, on the ptetM plasmid. To ensure directed mutagenesis of the chromosomal copy of *orf-1* in these strains, we constructed the plasmid pSK::*ST1*Δ*orf-1cat* (Fig. 2c), in which the complete *orf-1* coding region was deleted and replaced with a *cat* cartridge. This forced genetic recombination to occur via the flanking regions of *orf-1* that are present on the chromosome but absent from the ptetM plasmid (Fig. 2b). This strategy of mutagenesis with complementation in *trans* resulted in several erythromycin- and chloramphenicol-resistant transformants, irrespective of whether an intact copy of *orf-1* (MS11 ptetM::*orf-1*/Δ*orf-1cat*) or the *E. coli glmU* gene (MS11pH11::*glmU_E*/Δ*orf-1cat*) was present on the ptetM plasmid. PCR analyses and hybridization experiments confirmed that this time the chromosomal copy of *orf-1* was replaced by the *cat* cartridge (Fig. 7). The successful rescue of gonococcal *orf-1* mutants by complementation with the *glmU* gene located on the ptetM25.2 plasmid strongly suggests that ORF-1 shares GlmU functions.

UTP transferase enzyme activity in *N. gonorrhoeae*. To unequivocally identify the *orf-1* gene product as the gonococcal *glmU* homolog, we measured GlcNAc 1-P uridyltransferase activities in extracts of the gonococcus MS11-A42 and the constructed recombinants. As illustrated in Table 2, the level of enzyme activity in the *orf-1* mutant carrying an intact copy of the gene on the ptetM25.2 plasmid (MS11 ptetM::*orf-1*/Δ*orf-1cat*) exceeded that of the parent strain about fivefold, indicating that the cloned gene catalyzes the formation of UDP-GlcNAc. Remarkably, when the mutant carrying the *E. coli glmU* gene on the ptetM plasmid (MS11 ptetM::*glmU*/Δ*orf-1cat*) was included, an even 6-fold higher level of enzyme activity was measured, resulting in a 30-fold increase of enzyme activity compared with the parental strain.

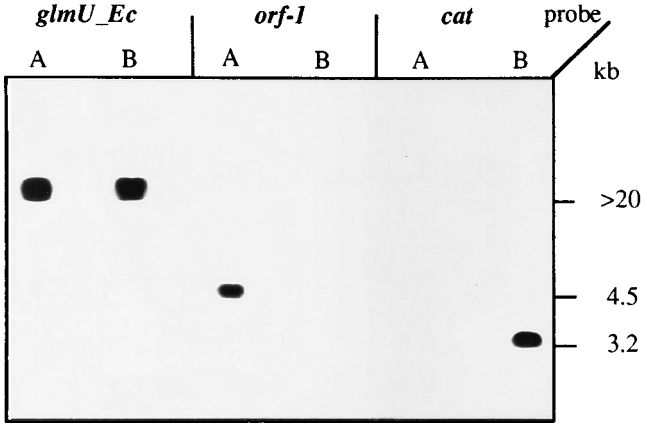

FIG. 7. Southern hybridization demonstrating the successful deletion of the chromosomal copy of *orf-1* in gonococcus strain MS11 carrying the *E. coli glmU* gene on the ptetM plasmid. *Cla*I-digested DNAs of strains MS11 ptetM::*glmU_Ec* (lanes A) and MS11 ptetM::*glmU_Ec*/Δ*orf-1cat* (lanes B) were blotted onto nylon membranes and hybridized with the peroxidase-labelled uridyltransferase genes of *E. coli* (*glmU_Ec*) and *N. gonorrhoeae* (*orf-1*), and the gene encoding the chloramphenicol acetyltransferase (*cat*). The *E. coli glmU* gene was detected on the ptetM plasmid (>20 kb) of both MS11 ptetM::*glmU_Ec* and MS11 ptetM::*glmU_Ec*/Δ*orf-1cat*. The deletion of the *orf-1* locus in the latter recombinant strain was demonstrated by the lack of hybridization of this mutant (lanes B) with the *orf-1* probe and the positive signal obtained with the *cat* marker. The smaller size of the *cat*-positive DNA fragment corresponds with the replacement of the *orf-1* gene with the smaller *cat* marker.

Thus, the *E. coli* gene appears to be expressed better or has a much higher intrinsic enzyme activity than its gonococcal counterpart. This may explain why only a moderate increase in GlmU activity was measured when the gonococcal gene was expressed in *E. coli*. It may be noteworthy that mutagenesis of *orf-1* did not result in a sialyltransferase-negative gonococcal phenotype. However, gonococcal GlmU activity in strain MS11 was inhibited up to 40% by 1.25 mM CMP-NANA (Table 2). This may explain the presence of a putative sialyl motif in ORF-1.

DISCUSSION

We identified a gonococcal gene encoding the enzyme GlcNAc 1-P uridyltransferase (EC 1.7.7.23) that catalyzes the conversion of *N*-acetylglucosamine 1-phosphate into UDP-*N*-acetylglucosamine, which is a key intermediate in the biosynthesis of LPS, peptidoglycan, and CMP-NANA. We propose naming this gene *glmU* in accordance with the nomenclature of the corresponding uridyltransferase in *E. coli*. The gene was identified as a *glmU* homolog on the basis of its extensive protein sequence homology with the uridyltransferases of *E. coli* and *B. subtilis*, the successful functional complementation of a gonococcal *glmU* mutation by the *E. coli glmU* gene, and measurements of GlcNAc 1-P uridyltransferase activity.

The gonococcal *glmU* homolog was identified as a result of our search for genes that contain DNA regions that are highly conserved among mammalian sialyltransferases and that participate in the recognition of the substrate CMP-NANA (6). The gonococcal *glmU* gene apparently falls into this category. The bacterial sequence contains a 33-amino-acid polypeptide sequence that has 15 identical and 8 conserved amino acid residues in common with the mammalian sialyl motif, including the highly conserved C-terminal sequence VGSKT. Despite this considerable homology, the gonococcal protein does not possess α2,3-sialyltransferase activity, as was demonstrated by the absence of such enzyme activity in *E. coli* expressing the gonococcal *glmU* gene and the unaltered LPS sialylation in the gonococcal *glmU* mutant complemented in *trans* with the *E. coli glmU* gene. Thus, the putative CMP-NANA binding site probably serves a different function. One possibility is that CMP-NANA is not the substrate for this enzyme but has a regulatory function. In *E. coli* the product UDP-GlcNAc forms the branchpoint in the synthesis of LPS, peptidoglycan, and *N*-acetylmannosamine, the last of which is converted into CMP-NANA (11, 35). CMP-NANA inhibits GlmU activity in *E. coli* (48). A similar allosteric inhibition of enzyme activity was observed for the gonococcal GlmU (Table 2). However, in contrast to *E. coli* and *N. meningitidis*, the gonococcus does not endogenously synthesize CMP-NANA, and thus we can only speculate that the presence of this putative regulatory cycle is not functional in gonococci. In meningococci, feedback inhibition of GlmU activity by CMP-NANA may affect the synthesis of polysialic capsule.

The gonococcal GlmU protein sequence shows a peculiar six-residue periodicity built around glycine residues. The sequence contains 44 glycine residues, of which 29 (65%) are located within one residue of a distance proportional to 6 and another 8 are found in the combination GXG. A similar six-residue periodicity is found for L, I, or V residues that often precede a glycine residue. Incomplete tandem hexapeptide repeats containing the sequence (L/I/V)(G/X)X(G/X)XX appear to be a characteristic of a number of bacterial acetyl- and acyltransferases (40). Whether the gonococcal GlmU enzyme has an additional function as an acetyltransferase has not been determined. In *E. coli*, the homologous enzyme catalyzes both the acetylation of glucosamine 1-P and the subsequent conversion of GlcNAc 1-P into UDP-GlcNAc.

In *E. coli* the *glmS* gene is located immediately downstream of and may be cotranscribed with the *glmU* gene (43). *glmS* encodes the enzyme glucosamine 6-phosphate synthetase, which catalyzes the formation of glucosamine 6-phosphate, which is then converted into glucosamine 1-P, which is the substrate for the *glmU* gene product. The *glm* genes are positioned between the *unc* operon and the *pst* operon which starts with *phoS* (43). This operon contains genes involved in the P_i uptake machinery and is regulated by the PHO regulon which is controlled by PhoR and/or PhoB (44). In the gonococci, the situation is different. At the C terminus, the gonococcal *glmU* gene is not followed by a *glmS* homolog but is flanked by an ORF that has extensive sequence homology to the first gene (*phnA*) of another member of the PHO regulon: the alkylphosphonate (Pn) operon which is involved in the uptake and utilization of carbon-phosphor compounds as a phosphate source (5, 32). Furthermore, upstream of *glmU* no ORF was detected within the first 150 bp of the putative start codon. Instead, the gonococcal *glmU* gene is flanked at its boundaries by inverted repeats containing the neisserial DNA recognition sequence, which are often components of transcriptional terminator sequences (13). The location of copies of the DNA uptake sequence between the putative promoter and the structural gene is unusual but has previously been reported (2).

Neisserial *glmU* is an essential housekeeping gene as demonstrated by the fact that *glmU* mutants could only be established in gonococci that were complemented with an intact copy of either the gonococcal or the *E. coli glmU* gene on the ptetM25.5 plasmid and that were grown in the presence of IPTG. Without derepression of the promoter activity, the bacteria carrying *orf-1* on the plasmid grew much more slowly, probably because of a shortage in the formation of the cell wall precursor UDP-GlcNAc. The requirement of IPTG for growth was much less pronounced for the mutant expressing the *E. coli glmU* gene. A possible explanation for this may be the finding that the level of *E. coli* enzyme activity was consistently higher than that of gonococcal GlmU activity, irrespective of whether the genes were expressed in the *E. coli* or gonococcal background. Whether this apparently higher level of enzyme activity is of biological significance with respect to, for instance, the growth rate of the bacteria is unknown, though the availability of UDP-GlcNAc may be a limiting factor in bacterial cell wall synthesis.

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