Implications of Phase Variation of a Gene (*pgtA*) Encoding a Pilin Galactosyl Transferase in Gonococcal Pathogenesis

Asesh Banerjee,[1] Rong Wang,[2] Sherry L. Supernavage,[1] Salil K. Ghosh,[1] James Parker,[3] Nisha F. Ganesh,[1] Peng G. Wang,[4] Sunita Gulati,[5] and Peter A. Rice[5]

[1]*Department of Biology, The Catholic University of America, Washington, DC 20064*
[2]*Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, NY 10021*
[3]*Department of Human Genetics, Mount Sinai School of Medicine, New York, NY 10029*
[4]*Department of Chemistry, Wayne State University, Detroit, MI 48202*
[5]*Department of Medicine, Section of Infectious Diseases, Evans Biomedical Research Center, Boston University Medical Center, Boston, MA 02118*

Abstract

The pilin glycoprotein (PilE) is the main building block of the pilus of *Neisseria gonorrhoeae* (gonococcus [GC]). GC pilin is known to carry a disaccharide O-glycan, which has an αGal attached to the O-linked GlcNAc by a 1–3 glycosidic bond. In this report, we describe the cloning and characterization of the GC gene, pilus glycosyl transferase A (*pgtA*), which encodes the galactosyl transferase that catalyzes the synthesis of this Gal–GlcNAc bond of pilin glycan. A homopolymeric tract of Gs (poly-G) is present in the *pgtA* gene of many GC strains, and this *pgtA* with poly-G can undergo phase variation (Pv). However, in many other GC, *pgtA* lacks the poly-G and is expressed constitutively without Pv. Furthermore, by screening a large number of clinical isolates, a significant correlation was observed between the presence of poly-G in *pgtA* and the dissemination of GC infection. Poly-G was found in *pgtA* in all (24 out of 24) of the isolates from patients with disseminated gonococcal infection (DGI). In contrast, for the vast majority (20 out of 28) of GC isolated from uncomplicated gonorrhea (UG) patients, *pgtA* lacked the poly-G. These results indicate that Pv of *pgtA* is likely to be involved in the conversion of UG to DGI.

Key words: *Neisseria gonorrhoeae* • pilus • DGI • poly-G tract • glycosyl transferase

Introduction

An estimated 650,000 cases of gonorrhea, caused by *Neisseria gonorrhoeae*, occur annually in the United States (1). Gonococcus (GC)* also causes additional complications such as pelvic inflammatory disease (PID), disseminated gonococcal infection (DGI), and ophthalmia neonatorum. Although most GC infections are uncomplicated and confined to genitourinary tract epithelium, in ∼1% of gonorrhea cases the bacteria gain access to systemic circulation and cause disseminated diseases (2). In addition, HIV-infected women exhibit an increased risk of contracting DGI (3, 4). However, not much is known about the mechanism of conversion of uncomplicated gonorrhea (UG) to the more complicated DGI.

Neisserial pili are filamentous surface structures that are polymeric fibers consisting mainly of pilin protein (*pilE* gene product). Pilus antigenic variation, resulting from frequent changes of the pilin amino acid sequence due to RecA-mediated exchanges between silent partial pilin genes (*pilS*) and the pilin expression locus (*pilE*), is important for GC to evade human immuno surveillance and may also play a role in determining tissue tropism (5–7). In addition, the GC pili undergo phase variation (Pv), the reversible interconversion between predominantly piliated and nonpiliated states (8, 9). A few mechanisms, some of which

Address correspondence to Asesh Banerjee, Department of Biology, The Catholic University of America, 620 Michigan Avenue NE, Washington, DC 20064. Phone: 202-319-5278; Fax: 202-319-5721; E-mail: banerjee@cua.edu

*Abbreviations used in this paper: DATDH, 2,4-diacetimido-2,4,6-trideoxyhexose; DGI, disseminated gonococcal infection; GC, gonococcus; HPAE-PAD, high performance anion exchange chromatography with pulse amperometric detection; IP, immunoprecipitation; IPTG, isopropyl-β-D-thiogalactopyranoside; LOS, lipooligosaccharide; MC, meningococcus; MS, mass spectrometry; ORF, open reading frame; *pgtA*, pilus glycosyl transferase A; PID, pelvic inflammatory disease; poly-G/C, homopolymeric tract of Gs or Cs; Pv, phase variation; UG, uncomplicated gonorrhea.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/2002/07/147/16 $5.00
Volume 196, Number 2, July 15, 2002 147–162
http://www.jem.org/cgi/doi/10.1084/jem.20012022

are similar to the antigenic variation, have been proposed to explain pilus Pv (10). Studies with human volunteers (11, 12), as well as with organ (13, 14) and tissue (15, 16) culture systems, have confirmed the important role of GC pili in establishing gonorrheal infection.

Several studies have clearly established that pilin of *N. gonorrhoeae* (17, 18) and the related pathogen *Neisseria meningitidis* (meningococcus [MC]; reference 19) are glycosylated. A high resolution x-ray crystallographic study (18) on GC strain MS11 (variant C30) reported the presence of an O-linked disaccharide Galα1–3GlcNAc–O (see Fig. 1) on the pilin protein. Pili of MC are glycosylated in the same region of the pilin molecule but instead of GlcNAc, they generally contain the unusual sugar, 2,4-diacetimido-2,4,6-trideoxyhexose (DATDH), as the O-linked residue (19). Also, unlike the GC glycan, the MC O-glycan is usually a trisaccharide, Galβ1–4Galα1–3DATDH–O. Nevertheless, other alternative forms of glycans, as well as a total lack of glycosylation, have been reported from some strains of both GC and MC (20, 21).

The homopolymeric tracts of Gs or Cs (poly-G/C) with seven or more consecutive G/C sequences are found within many structural genes of GC and MC (22), including those encoding glycosyl transferases involved in both lipooligosaccharide (LOS) biosynthesis (23–26) and capsule biosynthesis (27). Similar sequence features also occur in the promoter regions of genes encoding the outer membrane proteins Opc (28) and PorA (29), as well as in the signal peptide of PilC protein (30). These poly-G/C tracts mediate a high frequency (10^{-2}–10^{-4}) of reversible on/off switching of gene expression. This poly-G/C–mediated Pv of the aforementioned genes likely provides advantages to the bacteria in different niches of the human body. The MC *pglA* gene (31), which synthesizes the Galα1–3DATDH bond (similar to GC Galα1–3GlcNAc bond shown in Fig. 1) of MC pilin glycan, also contains a poly-G tract.

Here, we report the identification of a gene, pilus glycosyl transferase A (*pgtA*), which codes for the glycosyl transferase that forms the Galα1–3GlcNAc linkage of GC pilin glycan. We also provide a detailed analysis of the PgtA activity by various techniques of molecular genetics, biochemistry, and mass spectrometry (MS). We have found that in many GC, *pgtA* occurs in a phase-variable allelic form that carries a poly-G tract. However, in other strains and isolates, *pgtA* lacks the poly-G and is expressed constitutively without any variation. The implications of the presence and absence of *pgtA* variability on GC pathogenesis, particularly in relation to the causation of systemic versus uncomplicated gonococcal diseases, were examined.

Materials and Methods

Chemicals, Enzymes, Bacterial Strains, and Plasmids. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich and all enzymes were obtained from New England Biolabs, Inc. For all cloning, *Escherichia coli* XL-1Blue MRF′ (Stratagene) cells were used as the host. GC strains, plasmids, and oligonucleotides used in this study are listed in Table I. GC was grown using media and conditions as previously described (24, 32), and 2 μg/ml erythromycin were added for selection when needed.

Recombinant DNA Methods. Based on FA1090 genome sequence data, a pair of *pgtA*-specific oligonucleotide primers, PgtA1 and PgtA2 (Table I and see Fig. 2 A), was designed. λZapII vector–based (Stratagene) GC genomic libraries (25) of strain FA1090, MS11A, F62, and 15253 were screened by colony hybridization using the random prime-labeled PgtA1-PgtA2 amplicon according to published protocols (25, 33). Positive clones from the GC libraries were converted to their corresponding pBluescript II SK^- phagemids by using the manufacturer's in vivo excision protocol. Thus, phagemids ppgtA1, ppgtA2, ppgtA3, and ppgtA4 (Table I and see Fig. 2 A) were derived from F62, MS11A, FA1090, and 15253 libraries, respectively. Additionally, ppgtA5 was constructed by cloning the PgtA3-PgtA4 amplicon of FA1090 into EcoRV-BamHI–digested pBluescript II KS^+. The *pgtA* gene of ppgtA5 was knocked out by introducing *ermC′*, an erythromycin resistance cassette (24, 25). The resulting plasmid was designated as ppgtA-erm (Table I and see Fig. 2 A). ppgtA-erm DNA was transformed (34) into strains FA1090, MS11A, F62, and 15253 for disruption of *pgtA* in these GC. The transformants were selected on GC agar containing 2 μg/ml erythromycin. Later, the polar *ermC′* cassette was replaced with its nonpolar derivative, as we recently described (35). The knockouts were verified by PCR reactions using the aforementioned oligonucleotides by DNA sequencing of the PCR products and by Southern hybridization. The PCR conditions were the same as previously described (36). DNA was sequenced and oligonucleotides were synthesized by the Rockefeller University Protein/DNA Technology Center (New York, NY). Sequences of poly-G/C tracts were verified using a previously published method (23). The sequence analyses were performed using a Lasergene software package (DNASTAR). For sequence alignment, the Jotun-Hein algorithm of the MegAlign program of this package was used.

To examine Pv of *pgtA*, a few *pgtA*-FLAG reporter chimera were made (Table I and see Figs. 2 A and 8) using a pCMVTag4 vector kit (Stratagene). Several COOH-terminal translational fusions of the FLAG (DYKDDDDK) epitope tag with the GC *pgtA* open reading frame (ORF) were constructed. For this purpose, PgtA5-PgtA6 amplicons from the GC strains FA1090 (*pgtA* with 11-G poly-G) and F62 (without poly-G) were cloned into the three pCMVTag4 vectors using the SacI and EcoRV sites. As designed, the cloning of both FA1090 and F62 *pgtA* inserts in the pCMVTag4b produced "in-frame" PgtA–FLAG fusions, ppgtA–F1a and ppgtA–F2a, respectively, due to the merger of *pgtA* ORF with the functional ORF of FLAG. The cloning of the same FA1090 and F62 PgtA5-PgtA6 amplicons into pCMVTag4a or pCMVTag4c generated the "out of frame" PgtA–FLAG chimera, ppgtA–F1b and ppgtA–F2b, respectively, due to the fusion of the *pgtA* ORF with the nonfunctional ORFs of FLAG. All of the constructs were checked by appropriate PCRs, restriction analyses, and DNA sequencing. In addition, the reaction of commercially available anti-FLAG mAb M2 (Sigma-Aldrich) was used to distinguish the in-frame FLAG fusions from the out of frame ones by Western blotting (see below).

Extraction and Purification of Pilin Samples from GC Strains. Initially, pilin was extracted from GC according to previously published protocols (37–39) with minor modifications. Bacterial inocula from single, heavily piliated colonies were streaked densely onto 50 100 × 15–mm plates containing GC medium. 18–22 h later, the bacterial colonies were checked for piliation

Table I. *A List of GC Laboratory Strains, Oligonucleotide Primers, and Plasmids Used*

GC strains	Poly-G in *pgtA*	Isolated from	Site of isolation	Accession no.	Reference
FA1090	+, (11 G, on)	DGI	Cervix	AF485418	(73)
15253	+, (14 G, on)	DGI	Blood	AF485418	(74)
UUI	+, ~ (10 G, off)	DGI	Blood	AF485418	(75) and [a]
KH4318	+, ~ (11 G, on)	?	?	AF485418	?
M20	+, ~ (11 G, on)	?	?	AF485418	?
RT397	+, ~ (11 G, on)	?	?	AF485418	?
F62	—	UG	Female urethra	AF485419	(73)
MS11A/C	—	UG	?	AF485419	(38)
1291	—	UG	Male urethra	AF485419	(76) and [b]
R10	—	UG	Male urethra	AF485419	(38)
M13Ison	—	UG	?	AF485419	[c]
140Ison	—	UG	?	AF485419	[c]
Pgh3-2	—	UG	?	AF485419	(38)

Primers	Direction	59 Extension	Sequence	Position	Reference
PgtA1	F	-	CGGCGGAACAGGTTAAG	1,802 bp of ppgtA1 insert	Fig. 2 A
PgtA2	R	-	TCGATCAGGAAGCCGTTG	1,999 bp of ppgtA1 insert	Fig. 2 A
PgtA3	F	ATGATCGGATCC	AATTCGGTGACTACGGCTG	684 bp of ppgtA1 insert	Fig. 2 A
PgtA4	R	ATACCGGAATTC	ACCACCTTGTTTACGCCTT	2,279 bp of ppgtA1 insert	Fig. 2 A
PgtA5	F	ATGTAAGAGCTC	AATTCGGTGACTACGGCTG	684 bp of ppgtA1 insert	Fig. 2 A
PgtA6	R	ATACCGGATATC	TCGATCAGGAAGCCGTTG	1,999 bp of ppgtA1 insert	Fig. 2 A

Plasmids	Insert description	Reference
ppgtA1	3,356 bp F62 *pgtA* insert starting 1,138 bp upstream of start codon	Fig. 2 A
ppgtA2	MS11A *pgtA* DNA equivalent to 1,205–2,268 bp of ppgtA1 insert	Fig. 2 A
ppgtA3	FA1090 *pgtA* DNA equivalent to 1,445–3,345 bp of ppgtA1 insert	Fig. 2 A
ppgtA4	15253 *pgtA* DNA equivalent to 684–2,668 bp of ppgtA1 insert	Fig. 2 A
ppgtA5	FA1090 PgtA3-PgtA4 amplicon cloned in pBluescript II KS^+	Fig. 2 A
ppgtA5-erm	An XmaI-BsaWI *ermC′* (24) fragment cloned in AgeI site of ppgtA5 to knockout *pgtA*	Fig. 2 A
ppgtA-F1a	FA1090 PgtA5-PgtA6 amplicon cloned in pCMVTag4b. In-frame COOH-terminal fusion of FLAG epitope tag with the Pv^+ (11G) *pgtA*.	Fig. 2 A
ppgtA-F1b	Identical insert as above cloned in pCMVTag4c. Out of frame Pv^+ *pgtA*–FLAG fusion.	Fig. 2 A
ppgtA-F2a	F62 PgtA5-PgtA6 amplicon cloned in pCMVTag4b. In-frame fusion of FLAG with Pv^- *pgtA*.	Fig. 2 A
ppgtA-F2b	Identical insert as above cloned in pCMVTag4a. Out of frame FLAG fusion with the Pv^- *pgtA*.	Fig. 2 A

Restriction sites in the primers are underlined. ppgtA1, ppgtA2, ppgtA3, and ppgtA4 are from GC libraries made by cloning partially digested fragments of genomic *Tsp509*I in pBluescript II SK^-. Except in ppgtA1, DNA inserts are in the direction of *lacZ* in these plasmids. +, present; −, absent; ?, unknown; ~, approximate data; DGI, disseminated gonococcal infection; UG, uncomplicated gonorrhea; Pv^+, phase-variable *pgtA*; Pv^-, *pgtA* that expresses constitutively without any variation; F, forward or in the direction of *pgtA*; R, direction reverse to F. The number of Gs in each poly-G and expression status of *pgtA* are indicated in parentheses.
[a]Z. McGee, [b]M. Apicella, and [c]C. Ison, personal communications.

and harvested with cotton swabs into 50 ml ice-cold 0.15 M ethanolamine-HCl, pH 10.1. All subsequent operations were performed at 4°C. Bacteria were homogenized for 10 min at 2,500 rpm in a Sorvall Omnimixer. The suspension was centrifuged at 12,000 *g* for 30 min to remove the sheared bacteria. Pilin was precipitated by adding solid ammonium sulfate to 10% saturation, collected by centrifugation at 12,000 *g* for 45 min, and then the pellet was dissolved in 5 ml ethanolamine-HCl buffer. Insoluble contaminants were removed by centrifugation at 13,000 *g* for 60 min and the supernatant was dialyzed overnight against Tris-

saline, pH 8.0 (0.05 M Tris-HCI and 0.15 M NaCl). The crystals formed were collected by centrifugation at 12,000 *g* for 30 min and the pellet was dissolved in ethanolamine-HCl buffer.

Because such semipurified pilin preparations carry LOS contamination (39), we performed 16.5% tricine SDS-PAGE (40) for separating pilin protein from the LOS. Two identical gels of pilin samples were run in a Mini-protean (Bio-Rad Laboratories) apparatus. One of the two gels was subjected to silver staining (41) to check the separation of the two biomolecules. The other gel was used for the isolation of separated pilin. This gel was stained with reversible zinc stain (Bio-Rad Laboratories) and the bands of pilin were located. Each pilin band was then cut from the gel and destained. Thereafter, pilin was extracted from the gel slices by spinning the crushed slices through an Ultrafree-DA cartridge (Millipore). This purified pilin was subjected to 16.5% tricine SDS-PAGE analysis and silver staining. Although the stained pilin showed no trace of LOS at this stage, the pilin samples that were run in the parallel gel were subjected to one more cycle of gel purification to reduce the possibility of LOS contamination. These samples were examined by MS to confirm the absence of contamination. Later, these doubly purified pilin preparations were used for Western analysis and monosaccharide composition determination.

Determination of Galactosyl Transferase Activity. The enzymatic assessment for the galactosyl transferase activity of PgtA was performed using a standard radioactive galactosyl incorporation assay, which has been used in previous investigations of bacterial glycosylation (42–44). Using similar methods, the membrane fractions of *E. coli* XL1-Blue host cells harboring the plasmids containing the GC *pgtA* gene were examined for their ability to catalyze the incorporation of radioactive Gal because this fraction is known to be the source for bacterial galactosyl transferases. Similar membrane fractions from *E. coli* XL1-Blue strain carrying no plasmid or carrying only the cloning vector, pBluescript II KS^+, were used as controls. The galactosyl transferase activity of the *pgtA* knockout plasmid, ppgtA5-erm, was also measured.

Bacterial cultures from mid-log phase (OD_{600} = 0.8) were harvested and resuspended in 50 mM Tris-HCl, pH 8.0, containing 30 mM magnesium acetate and 2 mM dithiothreitol (buffer A). Bacteria were lysed using a sonicator cell disrupter (model W-220F; Heat Systems-Ultrasonics) equipped with a microtip. Five consecutive 10-s pulses at setting 7 were applied under ice-cold conditions. Unbroken cells and nuclear cell debris were then removed by centrifugation at 5,000 *g* for 10 min at 4°C. Membranes were collected as pellets from cell-free lysate by ultracentrifugation at 180,000 *g* for 1 h at 4°C and resuspended in buffer A. The protein content of each membrane fraction was measured using the DC protein assay (Bio-Rad Laboratories). Using membrane fractions containing 800 μg total proteins, each galactosyl transfer reaction was performed in a final volume of 100 μl containing buffer A and 45,000 cpm [^{14}C]UDP galactose (Perkin-Elmer) substrate. After incubating the samples for 1 h at 37°C, the incorporation of radioactivity into lipid intermediates was measured. 900 μl chloroform/methanol (2:1) was added to stop the reaction and extract the lipid-linked intermediates according to the method of Osborn et al. (44). The radioactivity incorporated into the intermediates was measured in a liquid scintillation counter (2200 CA TRI-CARB; Packard Instrument Co.). The enzymatic activity of the membrane fraction prepared from the culture carrying ppgtA5 plasmid was also measured after induction at mid-log phase (OD_{600} = 0.8) by adding 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h.

Western Blot Analysis. For the characterization of the *pgtA* mutation, ~100 ng wild-type MS11A pilin and ~500 ng MS11A*pgtA* pilin were added to wells in a 10% Bis-Tris NuPAGE gel (Novex) run in an electrophoresis chamber (XCell II; Novex). 10 μl SeeBlue (Novex) prestained marker was used as the mol wt standard and 10 μl of 1 mg/ml mouse laminin (Sigma-Aldrich) was added per lane as the positive control for monitoring GSL1-B4 and anti-αGal reactions. Electrophoresis was performed according to the manufacturer's protocols. Western transfer was then performed using the XCell II (Novex) module. After transfer, Immobilon-P (Millipore) polyvinylidene difluoride membrane was cut into three pieces. The first piece was treated with the mouse anti–GC pilin mAb 1E8/G8 (provided by M.S. Blake, Baxter Healthcare Corporation, Columbia, MD; reference 45), the second with the αGal-specific biotinylated lectin GSL1-B4 (Vector Laboratories), and the third with a human anti–αGal polyclonal Ab (provided by X. Chen, Wayne State University, Detroit, MI; reference 46). For each of these primary Ab/reactant reagents, goat anti–mouse IgG, streptavidin, or goat anti–human IgG was used, respectively, as the secondary Ab/reagent. The blots were developed using Western Blue (Promega) alkaline phosphatase substrate according to the manufacturer's instructions.

For the Western analysis of the PgtA-FLAG chimera, similar methodologies of electrophoresis and transfer as described above were used. However, the mAb used in this case was anti-FLAG M2 (Sigma-Aldrich). In addition, the positive control for M2 reaction was a firefly luciferase–FLAG fusion, pTag4lux–FLAG (Stratagene).

Accurate Molecular Mass Determination of GC Pilin Proteins by MS. This analysis was performed according to our previously published immunoprecipitation (IP)/MS protocol (47). In this experiment, the antipilin mAb, 1E8/G8 (45), was used for the IP of GC pilin. 5 μl semipurified preparation of pilin (containing ~5 μg protein) was incubated overnight with 200 μl hybridoma culture supernatant of mAb 1E8/G8 and 3 μl protein A/protein G plus agarose beads (Oncogene Research Products). For this incubation, a modified dilution buffer (140 mM NaC1, NOG [0.1% *N*-octyl-glucoside], 10 mM Tris-HCl, pH 8.0) was used. The beads were precipitated and washed twice with 500 μl ice-cold dilution buffer. The beads were then washed with 500 μl Tris buffer (10 mM Tris-HCl, pH 8) and 500 μl deionized distilled water, respectively. Lastly, pilin protein was extracted from the precipitated beads using 2 μl formic acid/water/isopropanol (1:4:4, vol/vol/vol), which contained saturated 4HCCA (α-cyano-4-hydroxycinnamic acid) and the mass calibrant, bovine insulin (Sigma-Aldrich). The molecular masses of pilin proteins were measured by a matrix-assisted laser desorption/ionization time of flight mass spectrometer (PerSeptive Biosystems).

Monosaccharide Composition Analyses of GC Pilin Glycans. This was performed by the high performance anion exchange chromatography with pulse amperometric detection (HPAE-PAD) method. The procedure was performed with a slight modification of a previously published HPAE-PAD protocol (48). A DX-500 HPLC workstation from the Dionex Corporation was used for this purpose. Purified pilin protein was subjected to TFA (2 N) hydrolysis and HCl (6 N) hydrolysis at 100°C for 4 h to release the sugars as monosaccharides. TFA hydrolysis yields accurate values for neutral sugars but not for amino sugars whereas the reverse is true for HCl hydrolysis (48). Therefore, both hydrolysis protocols were applied together in most monosaccharide analyses for the correct determination of sugar composition. During acid hydrolysis, GlcNAc and GalNAc are converted quantitatively to

GlcN and GalN due to deacylation and therefore are represented by two peaks in the PAD chromatograph, respectively. After each acid hydrolysis, the hydrolysate was purified by a passage through a Microcon-SCX (Millipore) cartridge, which is known to retain the amino acids. The hydrolysates were then passed through a PA-10 (Dionex) column in a DX-500 machine for monosaccharide analysis. The PA-10 column was attached to an amino trap guard column and an anion trap column (standard assembly for monosaccharide analysis of glycoproteins). The eluent was 18 mM NaOH with the flow rate of 1 ml/min. The experiments were performed at room temperature.

Southern Hybridization. The protocol used is a minor modification of our previous protocols (24, 49). The probe, a random prime-labeled PgtA1-PgtA2 amplicon (Table I and see Fig. 2 A), was made using the ECL kit (Amersham Biosciences) according to the manufacturer's instructions. GC genomic DNA was isolated as previously performed by Moxon et al. (50). BsrBI was used for the digestion of genomic DNA and Southern hybridization was performed on Hybond-N^+ nylon membranes (Amersham Biosciences) according to the manufacturer's manual.

Colony Immunoblotting for Examination of pgtA Pv. The protocol used for this experiment is similar to that described by Sambrook et al. (33). *E. coli* XL-1Blue cultures were grown in Luria-Bertani broth to log phase. Cultures were then diluted to obtain $\sim 10^3$–10^4 bacteria/ml and 10 or 20 μl of the dilutions were plated per spot on Luria-Bertani agar plates. The plates were incubated at 37°C with 5% CO_2 for 18–24 h. The colonies were transferred, lysed, and immunoblotted onto nitrocellulose filters strictly adhering to the protocol of Sambrook et al. (33). The colonies were detected using M2 anti-FLAG mAb and the Western Blue (Promega) detection kit.

Results

Cloning of pgtA from GC. The cloning of *pgtA* from GC was an offshoot of our previous work (25) involving the discovery of the LOS biosynthetic gene (*lgtG*) whose product forms the α1–3-galactosyl bond between the LOS β chain Glc and the second core heptose. Before we started the search for *lgtG*, it was known that the product of the *E. coli* LPS synthetic gene, *rfaG,* synthesizes a Glcα1–3Hep bond that is somewhat similar to the Glcα1–3Hep bond of GC LOS that joins the β chain to the core. To identify the GC gene involved in joining the β chain to the LOS core, we searched for homologues of *E. coli* RfaG in the GC genome. A TBLASTN (51) search of the GC strain FA1090 genome sequence (using the University of Oklahoma server) yielded two putative GC ORFs that showed a similar level of homology to RfaG (~30% homology over a stretch of 130 amino acids; unpublished data). One of the *rfaG* homologues from GC, *lgtG*, was found to be involved in the formation of the Glcα1–3Hep bond that joins the β chain to the core of LOS (25). However, the knockout of the other ORF (now designated *pgtA*) did not alter the LOS phenotypes of GC (unpublished data).

Later, a BLAST search on Genbank using GC ORF as the query yielded the RfpB peptide of *Shigella dysenteriae* (sequence data are available from Genbank/EMBL/DDBJ under accession no. AAC60480) as the best score (~50% identity over the full-length of both proteins; see Fig. 2 B). RfpB was known to form a Galα1–3GlcNAc bond in the LPS of *S. dysenteriae* (52, 53). It was also known that GC pilin carries an O-linked Galα1–3GlcNAc (18). Thus, we hypothesized that this GC ORF (*pgtA*) could be forming the Galα1–3GlcNAc bond of GC pilin glycan by transferring the Gal to the O-linked GlcNAc. Therefore, we named it pilin glycosyl transferase A. The DNA sequences of the *pgtA* genes from strains FA1090 and MS11A are available from Genbank/EMBL/DDBJ under accession nos. AF485418 and AF485419, respectively.

After this, a report describing MC *pglA* (31) was published that proposed that PglA synthesizes the Galα1–3DATDH bond of MC pilin glycan. The Galα1–3DATDH bond of MC pilin glycan is similar to the Galα1–3GlcNAc bond (Fig. 1) of GC pilin glycan in spite of the significant difference between the sugars GlcNAc and DATDH. We found that the sequence identity between MC PglA and GC PgtA is ~96% (Fig. 2 B), which indicates a very strong conservation.

By searching various finished and unfinished bacterial genome data banks, we identified several more ORFs that show homology to *pgtA* (unpublished data). Among these frames and of particular interest is a strong homologue from *Pseudomonas aeruginosa* that has 36% identity over the full-length of GC *pgtA* (Fig. 2 B).

Examination of Galactosyl Transferase Activity of PgtA. It has recently been shown that glycosyl transferase activities that modify several surface proteins of gram-negative bacteria, including flagellin and pilin, display strong sequence conservation with glycosyl transferases participating in LPS biosynthesis (31, 54). This indicates a general glycosylation machinery for the synthesis of all bacterial surface glycans that likely uses conserved intermediates. Thus, the various gram-negative surface glycosyl transferases generally display glycosyl transferase activity when cloned by plasmid vectors into *E. coli* host strains (42, 43). This cloned transferase activity, like the LPS transferases, resides in the membrane fraction of *E. coli* cells. Therefore, one well-known test for bacterial galactosyl transferase involves the assessment of radioactive incorporation from [^{14}C]UDP Gal into lipid intermediate carriers, usually undecaprenoyl phosphoryl acceptors, by the membrane fraction of *E. coli* (42). This assay has been used in the past for the characterization of several bacterial galactosyl transferases (42–44) and we adopted a similar approach to demonstrate the galactosyl transferase activity of PgtA.

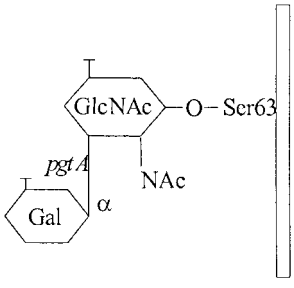


Figure 1. Current model of glycosylation of pilin of *N. gonorrhoeae*. Vertical bar on the right, pilin polypeptide; Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; Ser63, O-glycosylated serine at position 63 of GC pilin polypeptide.

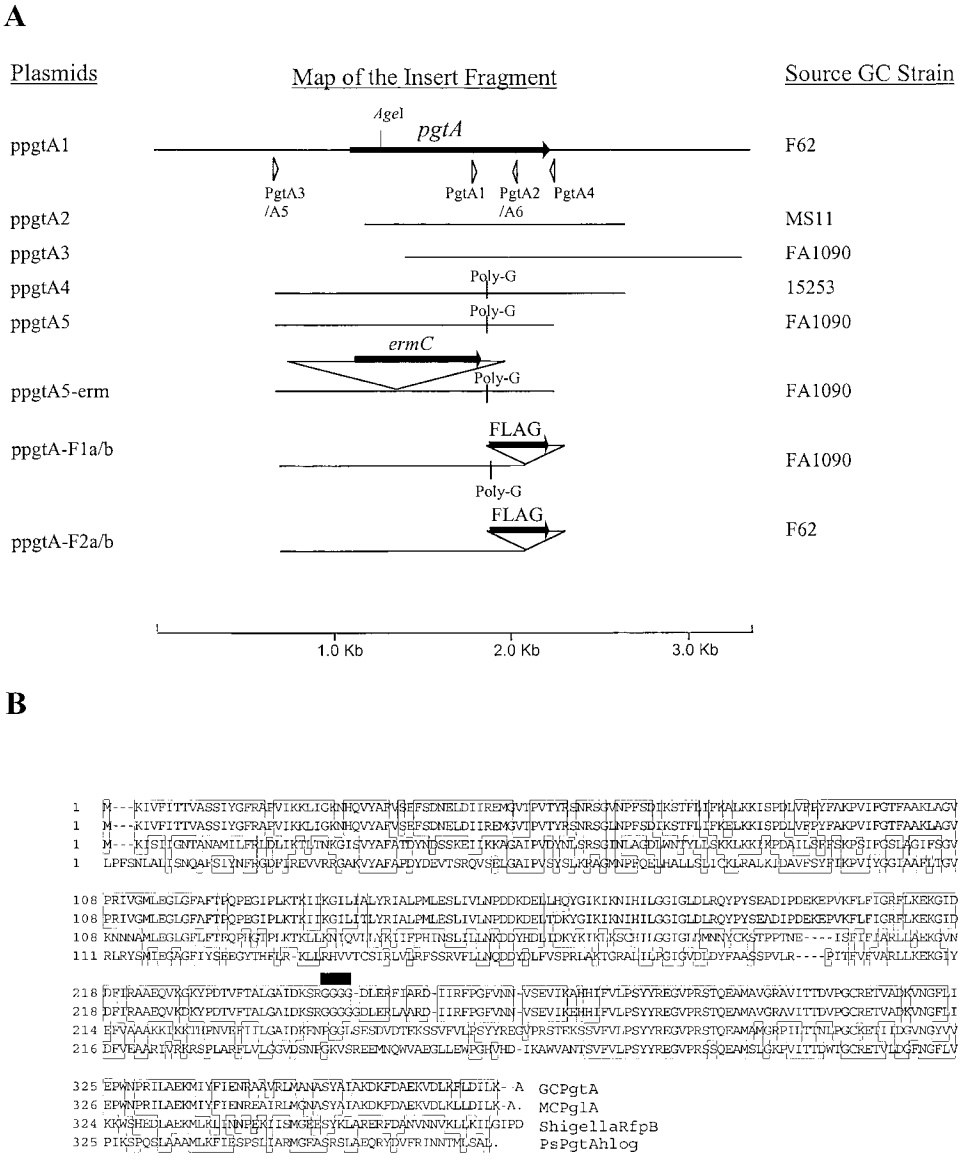


Figure 2. Molecular genetic characterization of the GC *pgtA* and its encoded activity. (A) Maps of the different GC DNA fragments that were cloned for analysis of the *pgtA* from various strains. Solid arrows, ORFs; vertical bars, poly-G tracts; open arrows, the location and direction of the oligonucleotide primers. (B) Alignment of PgtA homologues. Amino acid sequence of PgtA from GC strain FA1090 (GCPgtA) is compared with that of PglA from MC (MCPglA), RfpB of *S. dysenteriae* (ShigellaRfpB), and a functionally unknown ORF of *P. aeruginosa* (PsPgtAhlog). For this alignment, Jotun-Hein algorithm of the MegAlign program (DNASTAR) was used. The boxes represent sequence conservation. The tract of four consecutive glycines (residues 246–249) of PgtA, which correspond to the poly-G sequence, is marked by a thick solid line over it.

The Gal transferase activity of PgtA was examined in the background of the cloning host strain, *E. coli* XL1-Blue (Fig. 3). The activities of the membrane fractions of the XL1-Blue bacteria carrying the plasmids with a functional *pgtA* gene (ppgtA1 and ppgtA5) were found to be ~10-fold higher compared with that of the control hosts carrying no plasmid or the pBluescript II KS$^+$ vector. The bacteria with the cloned *pgtA* knockout (ppgtA5-erm) displayed a similar level of activity compared with the controls. Notably, all assays were performed using equal amounts of membrane fraction as well as identical reaction conditions (Materials and Methods). Both forms of *pgtA*, the constitutive (ppgtA1) and the phase-variable (ppgtA5), were tested. Moreover, to obtain a clearer understanding of the association of the Gal transferase with PgtA function, we decided to measure the galactosyl incorporation by the bacteria carrying ppgtA5 after the addition of IPTG. An induction of *pgtA* expression by IPTG was expected in this case because of the lack of a transcriptional stop between the Lac promoter and the *pgtA* promoter. A part of the *pgtA* mRNA would likely be transcribed from the inducible Lac promoter of the cloning vector, pBluescript II SK$^+$. Indeed, a roughly twofold increase in activity was observed after the IPTG induction (Fig. 3), further confirming the specific association of the Gal transferase activity with the PgtA function.

Lectin and Ab Reactions of MS11A and MS11ApgtA Pilin Proteins. Previously, by performing Western blots with MC pilin, Hamadeh et al. (55) observed that MC pilin reacts with the αGal-specific lectin GSL1-B4 as well as with anti-αGal human polyclonal Abs. We decided to test pilin proteins of GC strain MS11A and its isogenic *pgtA* mutant with these two reagents, particularly because no specific mAb is currently available that recognizes the αGal epitope of GC pilin glycan. Among the GC strains, MS11 was chosen for this analysis so that our data can be comparable to

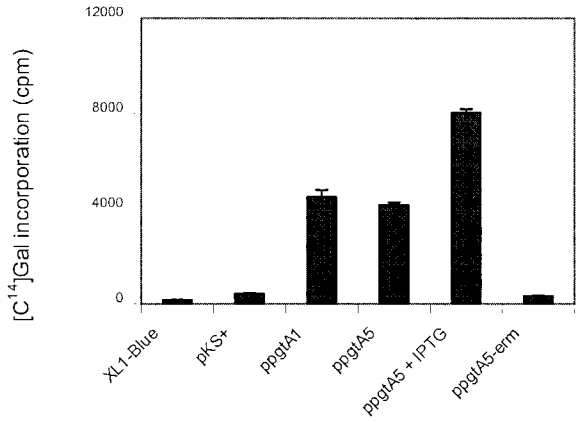


Figure 3. Galactosyl transferase activity in the membranes of *E. coli* XL1-Blue–expressing GC *pgtA*. The columns indicate the incorporation of radioactive galactosyl moiety from [^{14}C]UDP Gal into the lipid-linked intermediates catalyzed by different *E. coli* membrane fractions expressing various plasmids. *E. coli* XL1-Blue (without any plasmid) and its derivative with cloning vector pBluescript II KS$^+$ (pKS$^+$) were used as controls for this enzymatic assay. The same host strain with plasmids ppgtA1 and ppgtA5 were assessed for their ability to transfer Gal under the same condition. The specificity of galactosyl transferase activity of PgtA from ppgtA5 was also tested by induction with IPTG (5 mM), as part of this *pgtA* transcript is likely transcribed from the Lac promoter of the cloning vector pKS$^+$. The activity of ppgtA5-erm (*pgtA* knockout) was also evaluated. All assays were done in duplicate and standard deviations are shown by error bars (not visible on all columns).

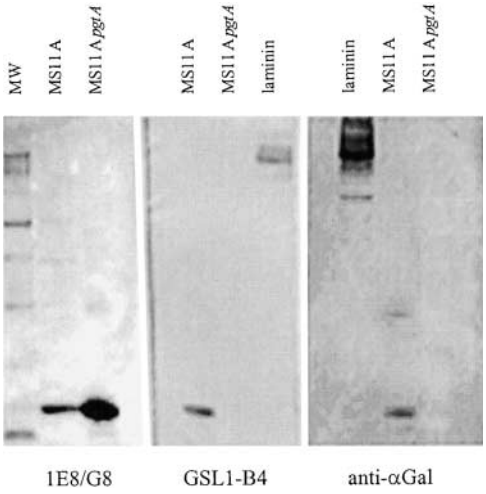


Figure 4. Western analysis of MS11A and MS11A*pgtA* pilins using antipilin mAb 1E8/G8, GSL1-B4 lectin, and polyclonal human anti–αGal Ab, respectively. Mouse laminin was used as a positive control for GSL1-B4 and anti-αGal reactions. Lanes marked MW contain SeeBlue prestained molecular weight marker (Novex).

the past studies of GC pilin glycan, which extensively used different variants of strain MS11. Also, the variant A of this strain (MS11A) is used as it produces a smaller LOS that runs farther separated from the pilin in SDS-PAGE and thus lessens the chance of LOS contamination of pilin during the gel extraction.

Western blots (Fig. 4) showed a specific reaction of GSL1-B4 with the wild-type MS11A pilin. This lectin did not react with the pilin of the isogenic *pgtA* mutant. Similarly, the anti-αGal human polyclonal Ab only reacted with the wild-type MS11A pilin and not with the MS11A*pgtA* pilin. The antipilin polypeptide mAb, 1E8/G8, was used as a control to monitor pilin proteins. Mouse laminin, well known to have αGal, was used as a positive control for the GSL1-B4 as well as for the anti-αGal reaction. In addition, neither the lectin nor the anti-αGal Ab reacted with LOS molecules of either MS11A or MS11A*pgtA* (unpublished data), further demonstrating the specific nature of this interaction.

MS of Pilin Proteins from MS11A and MS11ApgtA. IP/MS analysis was performed on pilin proteins obtained from MS11A and its isogenic *pgtA* knockout mutant. The major peak ([M+H]$^+$ ion) representing the wild-type pilin from MS11A had a molecular mass of 17,934.7 daltons (Fig. 5). The molecular mass of the corresponding peak from the isogenic *pgtA* mutant was 17,772.5 daltons. The mass difference (162.2 daltons) corresponded well with the mass of a hexose sugar molecule (162.1 daltons). Doubly charged peaks ([M+2H]$^{2+}$) were observed from both the MS11A (8,957.1 daltons) and MS11A*pgtA* (8,874.4 daltons) samples. The difference (82.7 daltons), when multiplied by 2, also yields a value (165.4 daltons) that corresponds well with a loss of hexose in the case of the mutant.

Additionally, the observed relative molecular mass of the pilin samples tallied well with the predicted molecular weight of pilin proteins derived from the amino acid sequences of MS11 pilins (18). The internal standard, human insulin peptide (M_r = 5,734.60), worked well in both spectra. Another peak (M_r = ~8,548) was observed not only in both spectra but also in the control (no pilin) spectrum containing only the reagents (unpublished data). Therefore, this peak likely comes from either the mAb used during the IP step or another reagent.

Monosaccharide Composition of Pilin Glycans of MS11A and MS11ApgtA. We determined the monosaccharide composition of the purified pilin proteins of GC strain MS11A and its isogenic *pgtA* mutant by the HPAE-PAD. The HPAE-PAD chromatograms are shown in Fig. 6. A substantial decrease in the relative Gal content of pilin is observed in the *pgtA* mutant when compared with the wild-type MS11A. This result would be expected from the postulated role of PgtA in transferring a Gal to the O-linked GlcNAc of GC pilin. However, we also observed other sugars present in chromatograms that cannot be accounted for by the current model of the disaccharide pilin glycan (Fig. 1). These observations possibly stem from the fact that the GC pilus glycan is likely to be more elaborate and complex than the current model.

Presence or Absence of Poly-G in pgtA of GC Laboratory Strains. DNA sequences of *pgtA* from the ppgtA plasmids and amplicons (Table I and Fig. 2 A) revealed the presence of a phase-variable poly-G tract region in the *pgtA* ORF of GC laboratory strains FA1090, 15253, UU1, KH4318, RT397, and M20. An 11-G poly-G was found in the *pgtA*s of all these strains except for 15253, which has a 14-G

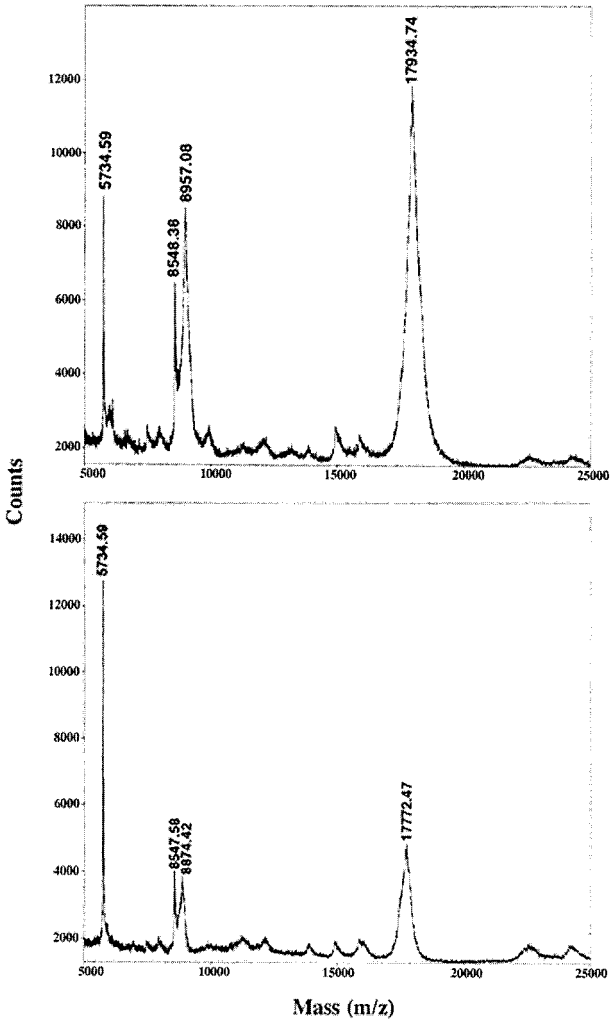


Figure 5. Matrix-assisted laser desorption/ionization time of flight MS study of pilins obtained from GC strain MS11A (top) and its isogenic mutant MS11*pgtA* (bottom). Bovine insulin peptide (molecular mass of 5,734.60 daltons) was added as the internal calibrant. Molecular masses of the major peaks are indicated.

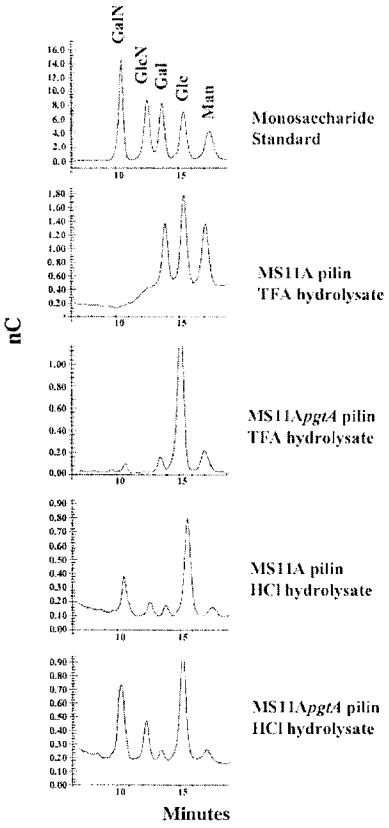


Figure 6. Monosaccharide composition analysis of pilins obtained from GC strain MS11A and its isogenic mutant, MS11A*pgtA*. The top chromatogram is of a standard mixture of monosaccharides containing 2 nmol of each component. The other chromatograms are hydrolysates of indicated GC pilins. Glc, glucose; Gal, galactose; Man, mannose; GalN, galactosamine; GlcN, glucosamine; C, coulomb.

poly-G (Fig. 7 A). Both 11-G and 14-G tracts correspond to the "on" frame of *pgtA*. In these *pgtA* alleles, if the number of Gs increases by one (frameshift by +1), a nonfunctional PgtA results with a premature termination of ORF at a position 91 amino acids less than the full-length PgtA. Similarly, if the number of Gs decreases to 10 (frameshift by −1), the ORF terminates prematurely at 126 amino acids ahead of the normal stop. The number of Gs in the poly-G of the *pgtA*s of strains FA1090 (these sequence data are available from Genbank/EMBL/DDBJ under accession no. AF485418) and 15253 was determined unambiguously because these alleles were sequenced from plasmids carrying cloned GC DNA inserts. Also, the on status of the 11-G *pgtA* of FA1090 has been confirmed by direct examination of PgtA-FLAG chimera (Fig. 8 A). In contrast, for strains UU1, KH4318, RT397, and M20, the numbers of Gs in the poly-G of *pgtA* (Table I) should be considered tentative, as these data numbers were obtained by sequencing PgtA1-PgtA2 amplicons after PCR. PCR is well known to produce shifts in a poly-G/C during amplification and the numbers of Gs in some of these poly-Gs might have changed from those actually present in the poly-G of the genomic DNA. For the same reason, the indicated on/off status of *pgtA* of these strains (Table I) should also be considered ambiguous because these designations are based on the determination of the number of Gs in the poly-G tract sequences after PCR.

Notably, the aforementioned phase-variable poly-G tract is not present in the *pgtA*s of GC strains MS11A, MS11C, F62, 1291, R10, M13Ison, 140Ison, and Pgh3-2. Among these strains, the *pgtA* DNA sequences of only MS11A (these sequence data are available from Genbank/EMBL/DDBJ under accession no. AF485419) and F62 were determined from plasmid clones. For the rest of the strains, *pgtA* sequencing was performed using PgtA1-PgtA2 amplicons. All of these *pgtA* alleles have nearly identical sequences that correspond to a nonvariable and constitutively on form of the gene. In these *pgtA*s, a GGGAGCGGGG sequence is found instead of the poly-G tract of the phase-variable allele (Fig. 7 A). The important differences found in the nonvariable allele are the presence of an A instead of the

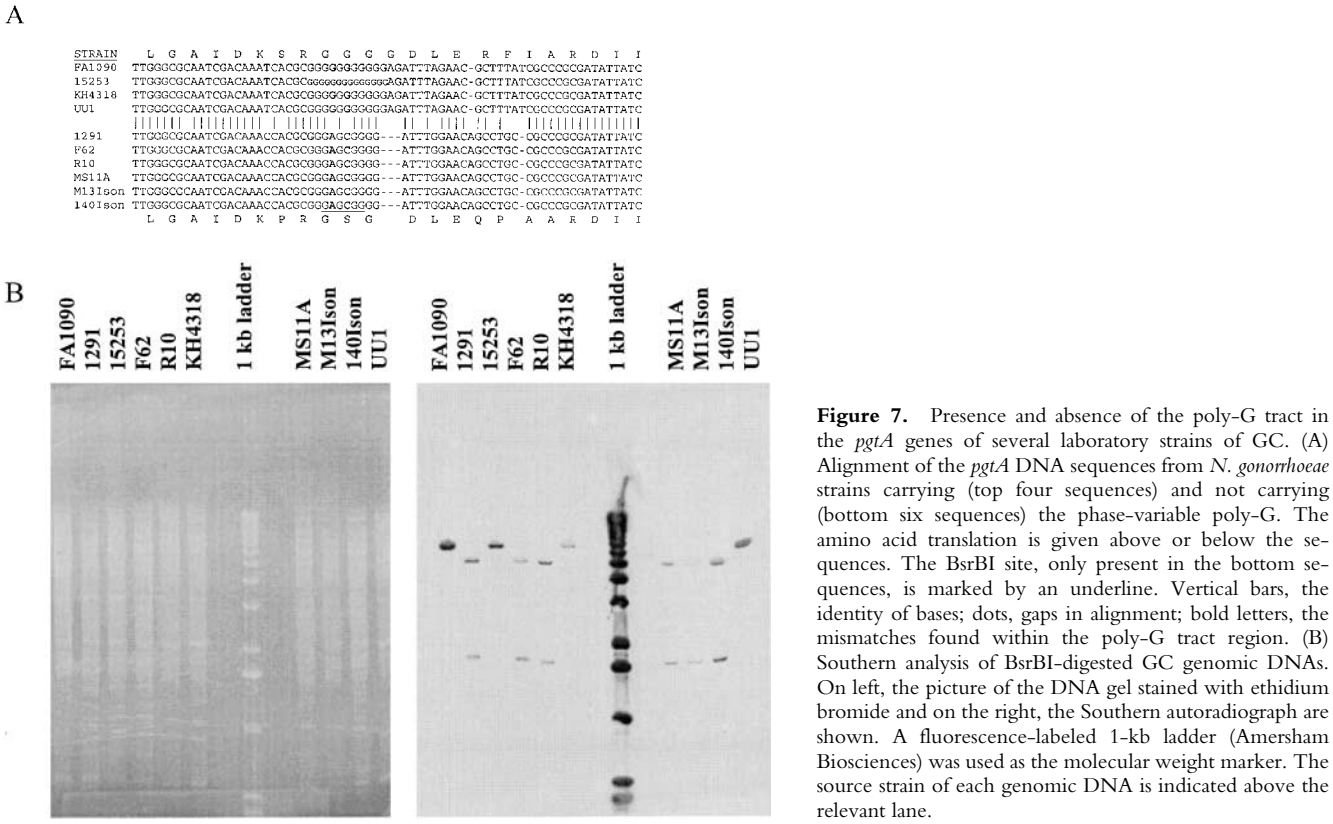


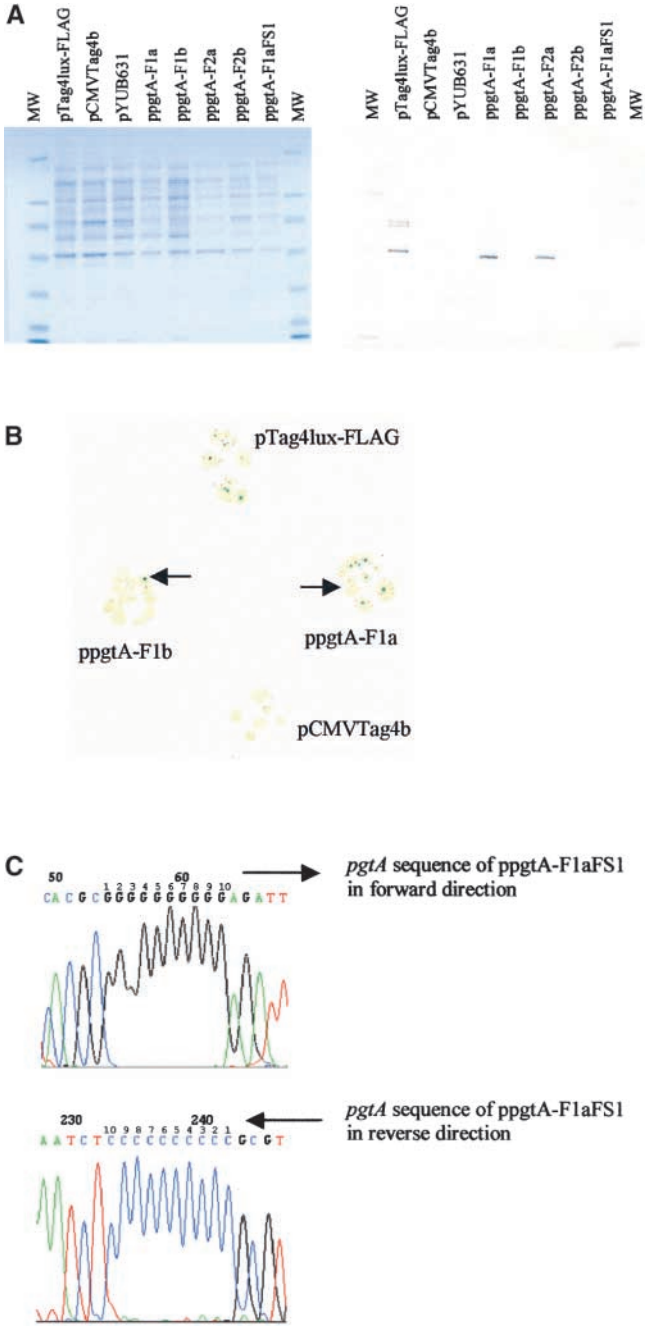
Figure 7. Presence and absence of the poly-G tract in the *pgtA* genes of several laboratory strains of GC. (A) Alignment of the *pgtA* DNA sequences from *N. gonorrhoeae* strains carrying (top four sequences) and not carrying (bottom six sequences) the phase-variable poly-G. The amino acid translation is given above or below the sequences. The BsrBI site, only present in the bottom sequences, is marked by an underline. Vertical bars, the identity of bases; dots, gaps in alignment; bold letters, the mismatches found within the poly-G tract region. (B) Southern analysis of BsrBI-digested GC genomic DNAs. On left, the picture of the DNA gel stained with ethidium bromide and on the right, the Southern autoradiograph are shown. A fluorescence-labeled 1-kb ladder (Amersham Biosciences) was used as the molecular weight marker. The source strain of each genomic DNA is indicated above the relevant lane.

fourth G, a C for the sixth G, and an absence of the last G of the poly-G. Near the poly-G, a few other changes were also observed between the two alleles as shown in Fig. 7 A. The rest of the sequence for the two types of *pgtA* is almost identical (unpublished data). The ORFs of both alleles are of similar length and apparently use the same start and stop codons. The *pgtA* that lacks poly-G carries an extra BsrBI restriction enzyme site (GAGCGG) in its GGGAG-CGGGG sequence.

Because the sequencing of G-rich DNA tracts is not always reliable, we verified the presence/absence of poly-G in *pgtA* by performing Southern hybridization of BsrBI-digested genomic DNAs of different GC strains. A labeled PgtA1-PgtA2 amplicon from strain FA1090 was used as a probe. In the Southern analysis (Fig. 7 B), the strains that were suggested to have GGGAGCGGGG showed two hybridizing bands indicating the presence of the extra BsrBI site. We observed that MS11A, F62, 1291, M13Ison, 140Ison, and R10 yielded the two-band pattern whereas FA1090, 15253, KH4318, M20, and UU1 showed a single band. This result confirmed the DNA sequencing data obtained from these strains.

Presence or Absence of Poly-G in pgtA of GC Clinical Isolates. We reviewed the clinical origin of the GC laboratory strains (Table I) that were divided into two categories based on the presence or absence of the poly-G tract in the *pgtA* gene. This analysis indicated an interesting correlation between the presence or absence of the poly-G with different disease phenotypes of gonococcal infection. F62, MS11A, 1291, R10, M13Ison, 140Ison, and Pgh3-2 (the strains lacking a poly-G in *pgtA*) were originally isolated from patients with uncomplicated infection. On the contrary, FA1090, 15253, and UU1 were obtained from DGI patients. This finding indicated the possibility that the DGI isolates bear a poly-G in *pgtA* whereas UG isolates do not. To further test this hypothesis, we examined the presence/absence of the poly-G tract in *pgtA* of many well-characterized patient isolates from three different gonococcal disease phenotypes: DGI, UG, and PID. PgtA1-PgtA4 amplicons from these isolates were analyzed by BsrBI RFLP as well as by DNA sequencing. The results showed a presence of the poly-G tract in all 24 (100%) DGI strains (Table II). In contrast, only 8 out of 18 (~44%) PID isolates (Table III) and 8 out of 28 (~29%) UG isolates (Table IV) contained the poly-G. It is noteworthy that in every case where poly-G was not found in *pgtA*, the GGGAGC-GGGG sequence was found instead. No other sequence has been observed to substitute poly-G in any GC strain or isolate. In comparison, the poly-G tract of *pgtA* was observed to be quite variable among the clinical isolates. The number of Gs in the poly-G varied from 9 to 20. However, the numbers of Gs present in the poly-G of *pgtA* (and the consequent on/off status of *pgtA*) bear no particular correlation with the different disease phenotypes, the sites of isolation, or the gender of the patients. Nevertheless, it must also be noted that the numbers of the Gs present in the poly-G tracts (as well as the indicated on/off phase status of *pgtA*) are only provisional. Again, this is for the same

reason (i.e., use of the PCR sequencing method that may shift the number of Gs in poly-G) as described previously in relation to the poly-G sequence determination of some GC lab strains. Furthermore, DNA was isolated from these clinical isolates after multiple in vitro passage and stocking of the bacteria. Thus, in these clinical isolates, both in vitro growth and DNA sequence determination by PCR sequencing may have caused changes in the number of Gs in the poly-G tract compared with the number present in organisms at the time and site of original isolation. Thus, the number of Gs for these isolates' *pgtA* poly-G may not always be accurate.



It is worthy to note that in our DGI collection, there are two isolates (8 and 13 in Table II) that are designated as having originated from more than one clinical site in the same patient. In these cases, GC was obtained from one of the indicated sites and not from all of the sites of the same patient. For example, in sample 13, B/U would mean that the GC tested was isolated either from the blood or the urethra of a particular patient. In this case, the clinical records show that the organisms were isolated from two sites of the patient's body but the bacterial stock only indicated the patient's name and not the site of isolation. Therefore, it was concluded that the organisms could have originated from any of the sites listed. It certainly does not mean that there are two separate isolates with the same number of Gs in poly-G of *pgtA* that were obtained from the blood and the urethra, respectively, from one individual.

Poly-G–mediated Pv of GC pgtA. The presence of poly-G tracts was observed in the *pgtA* genes of many GC strains and isolates. Because other poly-G carrying neisserial genes were previously found to be phase variable, the *pgtA* that had a poly-G was expected to show Pv. However, the *pgtA* lacking the poly-G (having a GGGAGCGGGG stretch instead) was predicted to be expressed constitutively without any variation based on Markov's model (22). Nevertheless, the latter GC-rich stretch may have also had a small possibility of displaying Pv because of two tandem short runs of Gs. Thus, it was necessary to verify the phase variability of both forms of *pgtA*.

Moreover, Pv of these alleles could not be tested using the polyclonal human anti–αGal Ab or the αGal-specific GSL1-B4 lectin because the colony blots using these re-

Figure 8. Poly-G–mediated Pv of the GC *pgtA* gene. (A) Western analysis of various PgtA–FLAG COOH-terminal fusion proteins expressed in *E. coli* XL-1 Blue host. On left, the Coomassie-stained gel shows the proteins present in the lysates of various *E. coli* hosts that carry different PgtA–FLAG fusions or controls. The corresponding Western blot, which was performed using anti-FLAG mAb M2, is shown on the right. The extreme lanes, marked MW, contain molecular weight marker. A firefly luciferase–FLAG fusion expressed from the plasmid, pTag4lux-FLAG (Stratagene), is the positive control for M2 reaction. The lysates from the cells carrying the vectors, pTag4b and pYUB631, are the two negative controls. ppgtA–F1a and ppgtA–F1b carry the in-frame and the out of frame fusions of FA1090 PgtA and FLAG, respectively. The in-frame and out of frame F62 PgtA-FLAG chimera are made by ppgtA–F2a and ppgtA–F2b, respectively. ppgtA–F1aFS1 is a putative phase variant (see below) of ppgtA–F1a resulting from a frameshift in *pgtA* poly-G. (B) The colony blot for the identification of potential phase variants of FA1090 *pgtA* cloned in *E. coli* XL-1 Blue host. The XL-1 Blue colonies carrying the positive control, pTag4lux-FLAG, are at the top spot. The negative control colonies, carrying pCMVTag4b vector, are at the bottom. The colonies of bacteria carrying the in-frame *pgtA*–FLAG (ppgtAF1a) and the out of frame *pgtA*–FLAG (ppgtAF1b) fusions are shown on the right and left, respectively. The potential phase-variant colonies are marked by arrows. (C) The DNA sequence of the poly-G tract of a potential phase variant (ppgtA–F1aFS1) obtained from the bacteria with the in-frame fusion (ppgtAF1a). The sequences from both DNA strands are shown with the leading strand data presented on the top. The direction of the transcription of *pgtA* for each sequence is indicated by an arrow. The G/Cs of the poly-G/C tract of *pgtA* are numbered in the direction of transcription.

Table II. *Examination of pgtA Poly-G Tract in GC Clinical Isolates Obtained from Patients with DGI*

GC isolate	Poly-G in *pgtA*	Site of isolation	Sex of patient	Reference
1	+, ~ (14 G, on)	B	F	(77)
3	+, ~ (14 G, on)	P	F	(77)
4	+, ~ (10 G, off)	B	M	(77)
5	+, ~ (14 G, on)	B	M	(77)
8	+, ~ (15 G, off)	B/C/P/R	F	(77)
10	+, ~ (13 G, off)	P	F	(77)
11	+, ~ (14 G, on)	U	M	(77)
13	+, ~ (15 G, off)	B/U	M	(77)
14	+, ~ (17 G, on)	C	F	(77)
21	+, ~ (17 G, on)	C	F	(77)
25	+, ~ (16 G, off)	C	F	(77)
27	+, ~ (14 G, on)	B	M	(77)
41	+, ~ (12 G, off)	SF	F	(77)
43	+, ~ (14 G, on)	SF	M	(77)
47	+, ~ (11 G, on)	C	F	(77)
48	+, ~ (11 G, on)	C	F	(77)
49	+, ~ (16 G, off)	C	F	(77)
Kb	+, ~ (11 G, on)	U	F	This study[a]
Tc-1	+, ~ (14 G, on)	SF	F	This study[a]
Tc-2	+, ~ (15 G, off)	?	F	This study[a]
Bu	+, ~ (17 G, on)	C	F	This study[a]
Aj	+, ~ (18 G, off)	SF	F	This study[a]
Dj	+, ~ (17 G, on)	SF	?	This study[a]
Jj	+, ~ (11 G, on)	?	F	This study[a]

C, cervix; B, blood; U, urethra; SF, synovial fluid; P, pharynx; R, rectum; M, male; F, female; +, present; ?, unknown; ~, approximate data; /, alternative sites of isolation (see Results). The number of Gs in each poly-G and expression status of *pgtA* are indicated in parentheses. It must also be noted that the on/off status of *pgtA* expression is tentative as this is predicted based on the numbers of Gs in the poly-G as determined by PCR sequencing.
[a]Strains taken from our collections that have not previously been reported.

Table III. *Presence and Absence of Poly-G Tract in GC Clinical Isolates Obtained from Patients with PID*

Isolate	Poly-G in *pgtA*	Reference
PID 1	—	(78)
PID 2	+, ~ (15 G, off)	(78)
PID 6-1	+, ~ (14 G, on)	This study[a]
PID 8	+, ~ (11 G, on)	(78)
PID 17	—	(78)
PID 18	—	(78)
PID 20	—	(78)
PID 22-1	—	This study[a]
PID 302	—	(78)
PID 305	+, ~ (15 G, off)	(78)
PID 305-1	+, ~ (15 G, off)	This study[a]
PID 332	—	(78)
PID 334	—	(78)
PID 335	—	(78)
PID 336-1	—	This study[a]
Ar	+, ~ (16 G, off)	This study[a]
Au	+, ~ (17 G, on)	This study[a]
Br	+, ~ (16 G, off)	This study[a]

All isolates are from cervix. +, present; −, absent; ~, approximate data. The number of previously published isolates are identical to that used in the cited reference. The number of Gs in each poly-G and expression status of *pgtA* are indicated in parentheses. It must also be noted that the on/off status of *pgtA* expression is tentative as this is predicted based on the numbers of Gs in the poly-G as determined by PCR sequencing.
[a]Strains taken from our collections that have not previously been reported.

agents yielded high background even for the *pgtA* mutants. These background reactions likely resulted from the nonspecific interactions of other surface antigens of GC, for example the αGal epitope of the alternative α chain of LOS. Therefore, we planned to test Pv of both alleles using a reporter-based approach that used a commercial mAb, knowing that such Abs were shown to work with colony blots.

A COOH-terminal FLAG epitope tag (DYKDDDK) was translationally fused with the *pgtA* ORFs in the following manner (also refer to Materials and Methods). First, FLAG fusions were made with the poly-G carrying FA1090 *pgtA* to obtain an in-frame (ppgtA–F1a) as well as an out of frame chimera (ppgtA–F1b). Similarly, in-frame (ppgtA–F2a) and out of frame FLAG (ppgtA–F2b) fusions were constructed with the F62 *pgtA*, which lacks a poly-G. All of the aforementioned constructs were expressed in *E. coli* XL1-Blue hosts. As we expected, all of the in-frame fusions reacted with anti-FLAG mAb M2 and all out of frame fusions failed to do so (Fig. 8 A). The positive control, a FLAG fusion with firefly luciferase (pTag4lux–FLAG) and the two negative controls, vectors pTag4b and pYUB631, behaved as we anticipated. The two negative controls were included to test if the unexpressed FLAG (lacking any promoter) of pTag4b produced any reaction with M2. pYUB631 does not contain any FLAG sequence.

The immunoblots of the XL1-Blue colonies having the in-frame fusion, ppgtA–F1a, yielded a few potential phase variants that failed to react with mAb M2 (Fig. 8 B). Similarly, potential phase variants showing positive M2 reaction were observed among M2 nonreactive colonies carrying the out of frame fusion (ppgtA–F1b). These potential variants arose with frequencies of $\sim 10^{-2}$. Four of the potential phase-variant clones that were obtained from the ppgtA–F1a colonies were sequenced from both DNA strands. All were found to carry 10 Gs in their poly-G tract, a number that is 1 G less (a −1 frameshift) than

Table IV. *Presence and Absence of Poly-G in GC Isolates from Patients with UG*

Isolate	Poly-G in *pgtA*	Sex of patient	Site of isolation
131[a]	—	M	U
227[a]	—	M	U
386[a]	—	M	U
405[a]	—	M	U
420[a]	—	M	U
490[a]	—	M	U
510[a]	—	M	U
517[a]	—	M	U
517109[a]	—	F	C
538[a]	+, ~ (17 G, on)	M	U
543[a]	—	M	U
543103[a]	—	F	C
545[a]	—	M	U
553[a]	+, ~ (18 G, off)	M	U
553630[a]	+, ~ (11 G, on)	F	C
575[a]	—	M	U
577[a]	+, ~ (10 G, off)	M	U
577123[a]	+, ~ (13 G, off)	F	C
582[a]	—	M	U
582125[a]	—	F	C
598[a]	+, ~ (14 G, on)	M	U
659[a]	—	M	U
PID 011[b]	+, ~ (9 G, off)	M	U
PID 022[b]	—	M	U
PID 023[a]	—	M	U
PID 032[a]	—	M	U
PID 036[a]	+, ~ (20 G, on)	M	U
PID 037[a]	—	M	U

Three-digit strains are from male patients with urethritis and corresponding six-digit (starting with the same three digits) strains are from their female contacts. The "PID 0" series strains are from male partners of PID patients. +, present; −, absent; M, male; F, female; U, urethra; C, cervix. The number of Gs in each poly-G and expression status of *pgtA* are indicated in parentheses. The reference used is this study. It must also be noted that the on/off status of *pgtA* expression is tentative as this is predicted based on the numbers of Gs in the poly-G as determined by PCR sequencing.

[a]Strains taken from our collections that have not previously been reported, and [b]strains reported in a study (78) that is also referenced in Table III.

that of the poly-G of the parent ppgtA–F1a. The sequencing results from both strands of one of these phase variants, ppgtA-F1aFS1, is shown in Fig. 8 C. The M2 reaction of the ppgtA–FS1 fusion protein is shown in Fig. 8 A. Notably, the blots of the ppgtA–F2a- and ppgtA–F2b-carrying colonies did not yield any potential variants (unpublished data), possibly due to a lack of poly-G in the F62 *pgtA*.

Discussion

In this study, we report the cloning and characterization of a GC pilin glycan biosynthetic gene that codes for a galactosyl transferase, which forms the α-glycosidic linkage between C3 of GalNAc and C1 of the αGal (Fig. 1). We named this gene *pgtA* (pilus glycosyl transferase A) adhering to the convention of other GC glycosyl transferases (24, 25, 56), particularly because we unambiguously demonstrated the enzyme encoded by it to be a galactosyl transferase. We first suspected this activity in this gene product because of its strong homology with the RfpB protein of *Shigella*, which has been shown to be the transferase that catalyzes the formation of a Galα1–3GlcNAc bond in LPS O-antigen. The use of human anti–αGal antibodies and GSL1-B4 lectin, and Western analyses of the pilin proteins of GC strain MS11A and its isogenic *pgtA* mutant, allowed a preliminary confirmation of our hypothesis. These two αGal-specific reagents reacted with wild-type MS11A pilin but not with the pilin of MS11A*pgtA*. In addition, MS studies demonstrated a clear loss of a hexose due to the *pgtA* mutation. HPAE-PAD–based monosaccharide composition analysis of pilin proteins provided the final confirmation of the proposed PgtA activity by demonstrating a quantitative loss of Gal in the *pgtA* mutant.

GC PgtA demonstrates very high homology (~95% identity) with MC PglA (31), which has been proposed to synthesize the MC pilin Galα1–3DATDH bond, a linkage analogous to that of Galα1–3GlcNAc of GC pilin. However, the proposed substrate molecules for the two enzymes (GlcNAc for PgtA and DATDH for PglA) are quite different. Also, it should be noted that the α1,3 galactosyl transferase activity of PglA has yet to be shown unequivocally. Nevertheless, the clear characterization of *pgtA*-encoded α1,3 galactosyl transferase supports the proposed activity of PglA (31). Interestingly, the most remarkable difference between *pglA* and *pgtA* is that based on the presence of poly-G. The former always seems to have Pv as it is found with poly-G all the time, whereas the latter can either undergo Pv or constitutively express the glycosyl transferase activity depending on the presence or absence of the poly-G. Furthermore, although no pathogenic implication of PglA has been indicated in MC pathogenesis yet, here we report a clear role for PgtA in GC pathogenesis. Therefore, a reevaluation of *pglA* might be needed to understand its role in MC pathogenesis. Lastly, it is also possible that PglA and PgtA may each act on both types of substrates (carrying DATDH or GlcNAc) and synthesize either type of pilin glycan depending on the specific background provided by different strains of MC and GC.

Notably, GC strains can be grouped into two categories based on the phenotype of phase variability of the *pgtA* gene. Sequence analysis demonstrated that *pgtA*s from certain GC strains and clinical isolates carried a poly-G tract, and that the number of Gs of this poly-G varied widely (between 9 and 20) from one isolate to another. In contrast, *pgtA* from other GC strains and isolates does not carry the variable poly-G tract but a GGGAGCGGGG sequence

instead, which differs from an analogous poly-G tract by only two bases. Still, the former allele of *pgtA*, but not the latter, was expected to be phase variable because poly-G/C tracts with seven or more G/Cs can mediate Pv (22). Using PgtA–FLAG fusions and colony blotting analyses, we demonstrated that the *pgtA* carrying poly-G is phase variable, but the allele lacking poly-G is expressed constitutively without any variation. Thus, GC *pgtA* is found in two mutually exclusive forms: one possessing and the other lacking Pv.

Our results indicate a possible significance for the presence or absence of the phase-variable poly-G tract in terms of GC pathogenesis. We observed that most GC associated with local infection only (uncomplicated inflammatory disease or PID in women) lack poly-G in *pgtA*. PID isolates may have the poly-G–bearing *pgtA* or the poly-G–lacking allele. However, all DGI-causing bacteria that we tested carried phase-variable *pgtA*. Poly-G–mediated Pv of *pgtA* may be advantageous for a GC isolate that disseminates for several reasons. One might be that anti-αGal IgGs are the most abundant Abs (~1% of total IgG population) in human sera (57) and turning off *pgtA* may help DGI isolates avoid these. In addition, Pv is known to increase the repertoire of neisserial antigens that often mimic human antigens and therefore may enhance tissue tropism of these bacteria (56, 58). Similarly, Pv of pilin glycan is likely to produce alternative glycoforms and therefore may lead to the expansion of potential host targets for GC that have disseminated. However, the nature of potential alternative pilin glycans having phase-variable *pgtA* in GC strains has yet to be determined, because such pilin glycans have never been characterized. Future experiments will require analysis of the pilin glycans strains that have phase-variable *pgtA* (such as GC strain FA1090).

Although previous studies have associated several phenotypes with DGI, the reason only a small percentage of untreated UG patients, and virtually no PID patients, develop DGI remains largely unknown (unpublished data). Clearly, serum resistance facilitates the systemic spread of GC by aiding survival in blood and can arise by several mechanisms (2). In addition, the arginine-hypoxanthine-uracil auxotype, porin 1A expression, and a peptidoglycan hydrolase gene (*atlA*) have been reported to correlate with DGI (59–61). Although each of these factors is found in a much higher proportion of DGI isolates than in UG or PID isolates, none shows absolute correlation with DGI. Our analysis showed that all (24 out of 24) DGI isolates tested carried phase-variable *pgtA*. DGI might be facilitated by multiple bacterial factors, none of which alone may be sufficient to cause dissemination.

Like phase-variable *pgtA*, *pgtA* lacking Pv may have its own advantages. This allele may help GC cause local infection because it is found in most UG (and PID) isolates and it may be relevant to examine whether a direct interconversion of these alleles is possible. Estimates from the 1970s suggest that ~1% of local gonorrhea proceeds to DGI (2). Although hypothetical, a DGI organism with a poly-G tract in *pgtA* could be directly selected from its counterpart that carries the corresponding nonhomopolymeric sequence, such a selection in vivo would require at least two point mutations when considering the normal frequency of mutational events. Nonetheless, the frequency of base changes (particularly for the second change, which would generate a full poly-G) in the GGGAGCGGGG sequence of the constitutive *pgtA* allele can be significantly higher than the normal mutational rate of GC, due to this stretch's close resemblance to a poly-G tract.

A comparative analysis of neisserial genomes (strains MC58, Z2491, and FA1090) by Saunders et al. (22) indicated the possible existence of a few genes that can be phase variable in one organism but not in another (62). Similarly, our study has shown *pgtA* to be a gene that exists in a phase-variable form in some strains of GC but not in others. To our knowledge, this is the first demonstration of a gene that can occur in both phase-variable and constitutive forms. This suggests the possibility that a gene might confer greater advantage to one strain of a pathogenic species as a "constitutive" gene, but to another strain of the same species as a "contingency" gene (63). The presence of the contingency allele of *pgtA* in DGI isolates would support the notion that these strains need greater adaptability in the more diverse and changing environment encountered at different systemic sites (e.g., blood, joints, skin, and occasionally the central nervous system and endocardium). In contrast, under perhaps the more "constant" environment of local genitourinary sites, the selection pressure for the contingency allele might be lessened. In this situation, a constitutive expression of the gene may be acceptable, or even preferred, if other benefits ensue.

Interestingly, from our search of existing databases, we observed poly-G tracts in numerous bacterial pathogens whose genomes have already been or are currently being sequenced (unpublished data). It is possible that poly-G–mediated Pv plays a role in the mechanism of pathogenesis in a number of bacteria. In addition, bacterial surface structures, particularly protein structures including the pili of other pathogenic bacteria, are increasingly being shown to be glycosylated (64–68). In particular, the *pgtA* homologue in *P. aeruginosa* is especially significant because both pilus and flagella from *Pseudomonas* are known to be glycosylated (64, 54). Future studies of bacterial surface glycans and their effects on Pv may yield important information concerning global pathogenic mechanisms.

Several types of glycosylation, present and absent, have been seen in different strains and variants of both GC and MC (17–21). Organizational variation of the *pgl* gene cluster among different MC strains (69, 70) also illustrates the degree of this diversity. Protein folding may influence glycosylation (71) and even a single amino acid change in a polypeptide may alter the glycosylation pattern (72). Diversification of glycosylation of neisserial pilus may stem from antigenic variation of pilin. Different pilin antigens may recruit separate sets of glycosyl transferases to produce distinct pilin glycosylation patterns. Therefore, synthesis of neisserial pilin glycan may involve yet unknown glycosylation enzymes. Notably, our HPAE-PAD analysis (Fig. 6) detected

novel sugars in MS11A pilin that were not described by our proposed model (unpublished data). A multiplicity of neisserial pilin glycoforms may arise because of involvement of phase-variable biosynthetic genes. As expected from this emerging diversity, the role of neisserial pilin glycans in pathogenesis of infection may take on an additional level of complexity.

We sincerely appreciate Dr. Emil C. Gotschlich's many invaluable contributions, including the idea of using the PgtA-FLAG chimera for studying Pv. We thank Dr. Ines Chen for several important reagents. We also thank Dr. John Golin for useful suggestions and editorial comments on the manuscript.

This work was supported by U.S. Public Health Service grants AI 47219 to A. Banerjee and AI 32725 to P.A. Rice.

Submitted: 5 December 2001
Revised: 8 April 2002
Accepted: 22 May 2002

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