Genetic characterization of pilin glycosylation in Neisseria meningitidis

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Pili of Neisseria meningitidis are a key virulence factor, being the major adhesin of this capsulate organism and contributing to specificity for the human host. Pili are post-translationally modified by addition of an O-linked trisaccharide, Gal(β1-4)Gal(α1-3)2,4-diacetimido-2,4,6-trideoxyhexose. In a previous study the authors identified and characterized a gene, pglA, encoding a galactosyltransferase involved in pilin glycosylation. In this study a set of random genomic sequences from N. meningitidis strain MC58 was used to search for further genes involved in pilin glycosylation. Initially, an open reading frame was identified, and designated pglD (pilin glycosylation gene D), which was homologous to genes involved in polysaccharide biosynthesis. The region adjacent to this gene was cloned and nucleotide sequence analysis revealed two further genes, pglB and pglC, which were also homologous with genes involved in polysaccharide biosynthesis. Insertional mutations were constructed in pglB, pglC and pglD in N. meningitidis C311 g3, a strain with well-defined LPS and pilin-linked glycan structures, to determine whether these genes had a role in the biosynthesis of either of these molecules. Analysis of these mutants revealed that there was no alteration in the phenotype of LPS in any of the mutant strains as judged by SDS-PAGE gel migration. In contrast, increased gel migration of the pilin subunit molecules of pglB, pglC and pglD mutants by Western analysis was observed. Pilin from each of the pglB, pglC and pglD mutants did not react with a terminal-galactose-specific stain, confirming that the gel migration differences were due to the alteration or absence of the pilin-linked trisaccharide structure in these mutants. In addition, antiseras specific for the C311 g3 trisaccharide failed to react with pilin from the pglB, pglC, pglD and galE mutants. Analysis of nucleotide sequence homologies has suggested specific roles for pglB, pglC and pglD in the biosynthesis of the 2,4-diacetimido-2,4,6-trideoxyhexose structure.

Keywords: pilin, glycosylation, glycosyltransferase, lipopolysaccharide, Neisseria meningitidis

INTRODUCTION

Pili of pathogenic Neisseria spp. are typical of a family of adhesins, type IV fimbriae, found in a wide range of Gram-negative pathogens. These long polymeric proteins protrude from the bacterial surface and have a crucial role in both colonization of the host and adhesion to host cells (Virji et al., 1991; McGee & Stephens, 1984). Although there are other accessory proteins, pili are composed primarily of thousands of subunits, called pilin. Typical of many surface proteins of pathogenic neisseriae, pili display both phase and antigenic variation (reviewed by Seifert, 1996). The gene encoding the

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pilin subunit, pilE, displays a very high degree of sequence variation, particularly towards the carboxy-terminal end of the protein. This sequence variation is mediated by unidirectional recombination with DNA from several silent, non-expressed copies of the gene pilS, and results in variation in adhesin function (Nassif et al., 1993; Virji et al., 1992, 1993). Pili of the pathogenic neisseriae are post-translationally modified (reviewed by Virji, 1997). Four different types of post-translational modifications have been described in detail. A phosphodiester-linked glycerol substituent has been reported at serine 93 of the pilin molecule (Stimson et al., 1995); covalently linked phosphorylcholine has been reported in N. meningitidis and N. gonorrhoeae (Wieser et al., 1998); a phosphate group has been reported at serine 68 in N. gonorrhoeae (Forest et al., 1999); and finally, pili of both N. meningitidis and N. gonorrhoeae are glycosylated. In N. meningitidis strain C311#3 a detailed structural study revealed that pilin is glycosylated at serine 63 with an unusual trisaccharide [Gal(\(\beta\)1-3)2,4-diaceitimido-2,4,6-trideoxyhexose] (Gal(\(\beta\)1-3)DATDH) (Stimson et al., 1995; see Fig. 8). Pili of the related pathogen N. gonorrhoeae are also glycosylated in the same region of the pilin molecule. However, the modification in N. gonorrhoeae is different from several silent, non-expressed copies of the gene pilS, which are post-translationally modified (reviewed by Stimson et al., 1995). Pili of both N. meningitidis and N. gonorrhoeae are post-translationally modified (reviewed by Virji, 1997). Four different types of post-translational modifications have been described in detail. A phosphodiester-linked glycerol substituent has been reported at serine 93 of the pilin molecule (Stimson et al., 1995); covalently linked phosphorylcholine has been reported in N. meningitidis and N. gonorrhoeae (Wieser et al., 1998); a phosphate group has been reported at serine 68 in N. gonorrhoeae (Forest et al., 1999); and finally, pili of both N. meningitidis and N. gonorrhoeae are glycosylated. In N. meningitidis strain C311#3 a detailed structural study revealed that pilin is glycosylated at serine 63 with an unusual trisaccharide molecule, Gal(\(\beta\)1-3)Gal(\(x\)1-3)2,4-diaceitimido-2,4,6-trideoxyhexose. The modification in N. meningitidis has been shown to be an O-linked disaccharide Gal(\(x\)1,3)GlcNAc (Parge et al., 1995), rather than the trisaccharide reported in N. meningitidis (Stimson et al., 1995). Marceau et al. (1998) have reported that a disaccharide, also proposed to be Gal(\(x\)1,3)GlcNAc, may be present in certain strains of N. meningitidis.

The precise function of glycosylation of pili has not been resolved. Since the glycosylated region of pili is proposed to be surface exposed and has a relatively conserved amino acid sequence, it has been suggested that glycosylation may constitute ‘bacterial cloaking devices against the host immune responses’ (Parge et al., 1995). Indeed, the work of Hamadeh et al. (1995) supports the binding of naturally occurring anti-Gal antibodies to the terminal galactose may interfere in complement-mediated lysis. Recent work by Marceau & Nassif (1999) has shown that the presence of the glycosylation at serine 63 can influence the amount of S-pilin (soluble pilin) produced. Defining the precise role of pilin glycosylation in host interactions awaits the elucidation of the genes involved in the biosynthesis of these carbohydrate structures. In a previous study we reported a gene, pglA, encoding a galactosyltransferase involved in the biosynthesis of the Gal(\(\beta\)1-4)Gal(\(x\)1-3)DATDH of N. meningitidis strain C311#3 (Jennings et al., 1998). In this work we present the identification and characterization of another biosynthetic locus involved in the biosynthesis of this structure.

### METHODS

**Strains, plasmids, media and growth conditions.** Meningococcal strains used in this study were MC58 (Virji et al., 1991), C311#3 and derivatives (Virji et al., 1993), or other strains as described by Scholten et al. (1994). Meningococcal strains were grown at 37 °C in 5% CO₂ on Brain Heart Infusion agar (BHI; Oxoid). BHI plates were made with 1% agar and supplemented with 10% Levinthal base (Alexander, 1965). Escherichia coli strain DH5α (Sambrook et al., 1989) harbouring various recombinant plasmids was cultured in LB broth or on LB plates containing 15% bacteriological agar (Difco; Sambrook et al., 1989). Ampicillin (amp) and kanamycin (kan) were used at a final concentration of 100 µg ml⁻¹.

**Recombinant DNA techniques and nucleotide sequence analysis.** Most recombinant DNA techniques were as described in Sambrook et al. (1989). Nucleotide sequence analysis was performed using the PRISM Dye Terminator Sequencing Kit with AmpliTaq DNA polymerase FS (Perkin Elmer) in conjunction with a model 373a automated sequencer (Applied Biosystems). Oligonucleotide primers were synthesized on a model ABI392 synthesizer (Applied Biosystems). PCR was essentially done as described by Saiki et al. (1988). Nucleotide sequence analysis was done using MacVector (Oxford Molecular).

**Southern blotting and hybridization.** Restriction endonuclease (Clal) digested genomic DNA was separated on 0.7% agarose gels and transferred to Hybond-N+ Nylon membrane (Amersham), essentially as described in Sambrook et al. (1989). Primers shown in Table 1 were used in the PCR reactions to amplify probes for pglB, pglC, pglD, and avtA (described in the legend to Fig. 1). The products were purified from agarose using a Qiagen gel extraction kit (Qiagen). The fragments were then DIG-labelled and hybridization was done using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim) as recommended by the manufacturer. All restriction endonucleases and ligases were obtained from New England Biolabs.

**Construction of knockout mutants of the pglB, pglC and pglD genes.** To construct insertional mutations in each of the pglB, pglC, and pglD genes, a kanamycin-resistance cassette (pUC4Kan: Pharmacia) was inserted into suitable unique

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**Table 1. Primers used for PCR of pglB, pglC, pglD and avtA genes**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Nucleotide position in AF14804</th>
</tr>
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<tbody>
<tr>
<td>PP1</td>
<td>5'-GCCGAAGACTTTGCATTCTC-3'</td>
<td>4351–4367</td>
</tr>
<tr>
<td>PP2</td>
<td>5'-GAAGACCTGTCGCCACGGCA-3'</td>
<td>4891–4909</td>
</tr>
<tr>
<td>PP3</td>
<td>5'-GGATAATCTGCGGCGAGA-3'</td>
<td>4036–4053</td>
</tr>
<tr>
<td>PP4</td>
<td>5'-AGTCGCGAATTGCTC-3'</td>
<td>478–495</td>
</tr>
<tr>
<td>PP5</td>
<td>5'-GAGGAGAGGATTTCCTC-3'</td>
<td>4333–4370</td>
</tr>
<tr>
<td>PP6</td>
<td>5'-ACAGCTTTATGCGGCCAAC-3'</td>
<td>1310–1327</td>
</tr>
<tr>
<td>PP7</td>
<td>5'-TCCGAAAGTCTATTTGGAA-3'</td>
<td>2923–2942</td>
</tr>
<tr>
<td>PP8</td>
<td>5'-GCGATGCTAACTGCGGTG-3'</td>
<td>5102–5122</td>
</tr>
<tr>
<td>PP9</td>
<td>5'-GCTGCTGAAAATTTGCGCC-3'</td>
<td>2730–2769</td>
</tr>
<tr>
<td>PP10</td>
<td>5'-AAGTGTGACCTTTCGGCGA-3'</td>
<td>3847–3866</td>
</tr>
<tr>
<td>PP11</td>
<td>5'-GTAAGCAGGCGCGCTCCTGC-3'</td>
<td>4551–4570</td>
</tr>
<tr>
<td>PP12</td>
<td>5'-GACGGCAAACACATCATG-3'</td>
<td>1915–1932</td>
</tr>
<tr>
<td>PP13</td>
<td>5'-TCAAACGGGGCCAAATCGG-3'</td>
<td>5681–5699</td>
</tr>
<tr>
<td>PP14</td>
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</tr>
<tr>
<td>PP15</td>
<td>5'-ACAGTTTATACCGCGGAC-3'</td>
<td>1310–1327</td>
</tr>
<tr>
<td>PP16</td>
<td>5'-GGTTTTCACCGAGCC-3'</td>
<td>6182–6199</td>
</tr>
</tbody>
</table>
restriction sites in the coding region of each gene (see Fig. 1). Previous work has demonstrated that this cassette has no promoter or terminator and will neither affect transcription nor have a polar effect (van der Ley et al., 1997; Jennings et al., 1995). The pgIB and pgIC knockouts were constructed by the digestion of pPPPCR5-42 (generated using the PCR primers PP5 and PP42 on C311; Table 1) and pPPPCR4-35 (generated using the PCR primers PP4 and PP35) with the restriction enzymes EagI and XcmI respectively. The restriction enzymes were then heat-inactivated for 20 min at 65 °C and the 5′ ends of the linearized plasmids were Klenow-filled. The plasmid containing the kanamycin-resistance cassette, pUC4Kan, was digested with HimII and the fragment containing the kanamycin-resistance gene was isolated and ligated to the linearized plasmids. The orientation of the insert was identified and plasmids containing the kanamycin-resistance ORF in the same orientation as the genes were selected and linearized with either EcoRI (in the case of the pgIB construct) or BamHI (for the pgIC construct). These plasmids were designated pPPPCR5-42kan and pPPPCR5-35kan for the pgIB and pgIC mutants respectively. The pgID insertional mutation was created by digesting pPPPCR12-18 (generated using the PCR primers PP12 and PP18) with XhoI and ligating the linearized fragment to the isolated SalI kanamycin-cassette-containing fragment derived from pUC4kan, resulting in the plasmid designated pPPPCR12-18kan. This plasmid was then linearized with BamHI. Transformation of N. meningitidis strain C311#3 with each of the pgIB, pgIC and pgID knockouts was carried out and recombinant strains were selected by growth on BH containing kanamycin (100 µg ml⁻¹).

**Fig. 1.** Restriction, plasmid and ORF map of pgIB, pgIC, pgID and avtA region. The initial sequence read (pGNMBA28F) from the TIGR genome sequencing project from which this locus was identified is represented as a thick black line at the top. The line labelled MC58 represents a restriction endonuclease map of the GenBank entry AF014804. The open arrows beneath the line indicate the orientation and location of the ORFs identified in the sequence. Below the ORFs the thick black lines represent the plasmids constructed during this work. The vectors of these plasmids are represented by boxes: unfilled for pT7Blue (Novagen) and black-filled for pCRScript (Stratagene). The restriction endonuclease sites shown on these plasmids indicate where the kanR cassettes were inserted. Rectangular boxes above the MC58 line represent the PCR products used as probes in Fig. 7. The pgIB, pgIC, pgID and avtA probes were constructed from PCR products utilizing primers PP35 and PP30, PP24 and PP11, PP15 and PP17, and PP27 and PP50, respectively.

**Pilin isolation from the pgIB, pgIC and pgID knockout mutants.** Pilin from each of the pgIB, pgIC and pgID knockout mutants was isolated based on the method described by Wolfgang et al. (1998). Briefly, cells were collected from five heavily streaked plates, resuspended in 800 µl 0.15 M ethanolate pH 10.5, vortexed vigorously for 60 s and centrifuged at 14000 g for 15 min. The supernatants were then collected, heat-killed at 56 °C for 30 min, and 200 µl of 4X loading dye added. The pilin from each strain was then separated in SDS-PAGE and semi-purified according to molecular mass, by electroelution (Bio-Rad).

**Production of antisera specific for the glycosylated pilin of C311#3 and C311#3pgIA.** Antisera directed against the pili expressed by C311#3 and C311#3pgIA were raised in Lop/New Zealand White rabbits as follows. Cells collected from plate cultures of each strain were resuspended in PBS at a density of 10⁹ c.f.u. ml⁻¹, vortexed for 1 min to shear the pili, and centrifuged at 14000 g for 15 min. The resulting supernatants contained similar amounts of semi-purified native pilin as confirmed by SDS-PAGE and Western analysis using the pilin-specific monoclonal antibody SM1. Each preparation of pilin was combined with an equal volume of adjuvant (MPL + TDM + CWS; Sigma M6661) to a final concentration of approximately 2.5 mg ml⁻¹ for inoculation into rabbits. Each rabbit received five 0.4 ml inoculations at intervals of 2 weeks, and blood was harvested by cardiac puncture 6 weeks after the final inoculation.

Using a modification of the method of Gruber & Zingales (1995), these sera were absorbed against meningococcal cells to enhance their specificity for glycosylated pili. Briefly,
RESULTS

Identification, cloning and sequencing of pglB, pglC, pglD and adjacent regions in strain MC58

In order to identify novel genes involved in pilin glycosylation, 400 random genomic sequences derived from a random plasmid library of N. meningitidis strain MC58 (The Institute for Genomic Research) were used to search against the combined GenBank/EMBL databases using the program BLASTX (Altschul et al., 1997). The search results were examined to identify matches with genes involved in polysaccharide biosynthesis. One of these sequences, GNMB28F (a read of 359 bp corresponding to the region 4351–4909 in the sequence deposited under accession number AF014804), showed a high level of similarity with the product of the trsG gene of Yersinia enterocolitica (Skurnik et al., 1995), and other genes involved in the biosynthesis of either lipopolysaccharide or capsular polysaccharide (see Table 2).

To further investigate this gene and its adjacent regions a cosmid library derived from strain MC58 was screened using a probe (designated pPPPCR1-2, generated using the PCR primers P1 and P2) derived from the GNMB28F sequence. One of the positive clones from this library, pC4, was chosen for further analysis. The cloned region was sequenced on both strands. Analysis of 6.5 kb of nucleotide sequence adjacent to the GNMB28F region revealed ORFs in the same orientation and in close proximity. These ORFs were designated pglB, pglC, pglD and avtA using the nomenclature of Jennings et al. (1998). The non-coding regions between these genes were small: 133 bp between pglB and pglC, 48 bp between pglC and pglD, and 56 bp between pglD and avtA, suggesting that they may be transcriptionally linked. The G+C contents of the ORFs were 57.4 mol% for pglB, 56.4 mol% for pglC, 56.5 mol% for pglD and 57.1 mol% for avtA. These figures are within the expected range cited for biosynthesis genes in N. meningitidis (49–56%, Morse & Knapp, 1992; Hoke & Vedros, 1982). Presumptive ATG start codons were observed for all ORFs. The putative stop codon for pglB, pglC and avtA was TAA and for pglD was TGA.

The homologies found with these genes are displayed in Table 2 and described below.

pglB. The pglB ORF is 1242 nt in length, starting at nucleotide 556 of the determined sequence (GenBank entry AF014804) and terminating at nucleotide 1797. The putative PglB protein is 414 aa in length and has a calculated molecular mass of 44.5 kDa. A BLASTX homology search (Altschul et al., 1997) revealed two regions of distinct homology within the pglB ORF.

The first region, consisting of the N-terminal 201 aa of PglB, had highest similarity with a family of presumed glycosyltransferases. The highest scores were to a group of putative transferases from Campylobacter that were first identified as necessary for LPS biosynthesis (50/63% identity/similarity: Wood et al., 1999; Fry et
Identification of proteins homologous to the products of Table 2.

<table>
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<tr>
<th>Putative protein</th>
<th>Identity/Similarity</th>
<th>Organism</th>
<th>Accession no.</th>
<th>Proposed function</th>
</tr>
</thead>
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<td>PglB (414 aa, 44.5 kDa, pI 9.41)</td>
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<tr>
<td>First 200 aa</td>
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<td></td>
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<tr>
<td>YvfC</td>
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<td>CAA96480</td>
<td>Glycosyltransferase</td>
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<td>AAC46096</td>
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<tr>
<td>Last 200 aa</td>
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<td>UDP-N-acetylgalactosamine acyltransferase</td>
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<td>Thermus aquaticus</td>
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<td>Aspartate aminotransferase (EC 2.6.1.1)</td>
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</table>

al., 1998). These genes have recently (Szymanski et al., 1999) been shown to be involved in flagellin glycosylation (see Discussion and Table 3). There is also similarity to genes involved in O-antigen synthesis in Anaabaena sp. (Korolik et al., 1997) and capsule biosynthesis in Staphylococcus aureus (Sau & Lee, 1996; Sau et al., 1997a, b). The activity of this family of genes is based upon similarity to RfbP of Salmonella typhimurium (Wang & Reeves, 1994, 1996), which acts to transfer galactose to an undecaprenol phosphate in the first steps of O-polysaccharide biosynthesis. The second region of homology consists of the C-terminal 166 amino acids (from amino acid 248). This region is homologous to a family of acetyltransferases. The highest similarity with this region is LpsB of Caulobacter crescentus (41/60%), which is believed to be an UDP-N-acetylgalactosamine acetytransferase involved in LPS biosynthesis (Awram & Smit, 1998). In common with this family of genes, this area of similarity contains an isoleucine patch which is speculated to play an important structural role in the function of these enzymes (Dicker & Seetharam, 1992). The two regions of the putative PglB also have distinct G+C contents, the N-terminal region having a G+C content of 52 mol% and the C-terminal region a G+C content of 62.2 mol%.

**pglC.** The second ORF, pglc, is 1176 nt in length, starting at nucleotide 1930 and ending at nucleotide 3105. The putative PglC protein is 392 aa in length and has a calculated molecular mass of 43.4 kDa. PglC is most similar (38/53%) to a DegT homologue in Methanobacterium thermophilum (Smith et al., 1997). This family of genes, although once thought to play a regulatory role, are now proposed to be aminotransferases (Thorson et al., 1994). The putative PglC has high similarity with a number of pyridoxal-binding dehydrases and aminotransferases and contains the aminotransferase class III pyridoxal-phosphate attach-
any saccharide biosynthesis. The position of prestained low-molecular-mass marker (21.5 kDa, Bio-Rad) is indicated by the arrowhead to the left of the figure.

**Fig. 2.** Analysis of the migration of pilin and LPS of the C311#3 derivatives. (a) Western blot analysis of the migration of pilin isolated from C311#3, C311#3gale and C311#3pglB, pglC and pglD mutants. Pilin was identified by the use of monoclonal antibody SM1 specific for class I pil of *Neisseria*. (b) Silver-stained gel showing the relative migrations of LPS of C311#3 and derivatives. Sample are labelled at the top of the figure.

**pglD.** The third ORF, pglD, begins at nucleotide 3153 and terminates at nucleotide 5063. This constitutes an ORF of 1911 bp and translation yields a peptide of 637 aa with a calculated molecular mass of 71 kDa. The putative PglD protein is highly similar to a family of proteins proposed to be dehydratases involved in LPS and O-antigen biosynthesis. It is most similar (55/72%) to TrsG of *Yersinia enterocolitica* (Skurnik et al., 1995).

**avtA.** The last ORF starts at nucleotide 5119 of the determined sequence and is 1293 bp in length, terminating at nucleotide 6441. The putative AvtA protein is 431 aa long, has a calculated molecular mass of 47.1 kDa and is homologous (46/65%) with AvtA from *E. coli* (Sofia et al., 1994). AvtA is an alanine-to-valine aminotransferase and has not been implicated in any saccharide biosynthesis.

**Construction and analysis of pgl::kan mutant strains.**

In order to determine whether pglB, pglC or pglD played a role in either LPS biosynthesis or pilin glycosylation, mutants were constructed by the insertion of a kanamycin-resistance (kan^R^) cassette into restriction endonuclease sites present in each of the coding regions (see Fig. 1). These insertional mutant constructs were then used to transform strain C311#3, a meningococcal strain with a well-characterized pilin structure, so that the inactive alleles were transferred to the chromosome by homologous recombination. The presence of the inactive allele was confirmed, both by Southern hybridization and by PCR of the mutated alleles (see Methods; results not shown).

**LPS phenotype of pglB, pglC and pglD::kan mutant strains.** Many of the genes involved in meningococcal LPS biosynthesis have been described (Jennings et al., 1999). However, there is still the potential for novel structures to be identified. To determine whether pglB, pglC or pglD affected LPS biosynthesis, the LPS of the mutant derivatives was analysed by SDS-PAGE. This revealed that there were no alterations in migration of the LPS molecule isolated from any of the mutant strains when compared to the parental strain and the control strain C311#3galE::kan (Fig. 2b). These results indicate that neither pglB, pglC or pglD is involved in LPS biosynthesis.

**Analysis of the migration of pilin from the pglB, pglC and pglD::kan mutants of strain C311#3.** Since a role for pglB, pglC or pglD in LPS biosynthesis could not be detected, we sought to test the alternative hypothesis that these genes were involved in pilin glycosylation. The structure of the trisaccharide moiety was determined in the meningococcal strain C311#3 and found to be Gal(β1-4)Gal[α1-3]DATDH (Stimson et al., 1995). Analysis of the previously described pglA and galE mutants of C311#3 demonstrated that modifications of the trisaccharide resulted in altered pilin migration in SDS-PAGE (Jennings et al., 1998). Analysis of pilin of the pglB, pglC and pglD mutant derivatives of C311#3 showed that in all three cases, the pilin migrated further than those from the parent strain (Fig. 2a). This observation suggested that pglB, pglC and pglD may affect pilin glycosylation.

**Sequencing of the pilE gene of the pglB, pglC and pglD::kan mutants of strain C311#3.** The gene encoding the pilin subunit, *pilE*, displays a high degree of sequence variation, particularly towards the carboxy-terminal end of the protein (Seifert, 1996). Alterations in the sequence of *pilE* result in alterations in pilin migration in SDS-PAGE. Therefore, the *pilE* genes of the pglB, pglC and pglD::kan mutants of C311#3 were sequenced and the sequences compared with the *pilE* sequence of the parental strain C311#3 (GenBank accession number L22639). The sequences (results not shown) confirmed that there was no difference in the *pilE* genes of the mutants compared to the parental gene, indicating that the differences observed in pilin migration from these mutants must be due to alterations in post-translational modifications.

**Pilin of the pglB, pglC and pglD mutants is not detected by a terminal galactose-specific method.** In order to determine whether the change in pilin migration was due to an alteration in the trisaccharide modification, as opposed to one of the other known pilin modifications, we examined the pglB, pglC, and pglD mutant derivatives using a terminal-galactose-specific detection system (see Methods). Semi-purified pilin (see Methods) extracted from C311#3, and its galE, pglA, pglB, pglC and pglD mutants, were separated by SDS-PAGE and transferred to PVDF membrane. Pilins containing terminal ga-
lactose moieties were then selectively detected by the use of biotinamidocaproyl hydrazide following galactose oxidase treatment, which creates aldehyde groups specifically on terminal galactose residues, resulting in the incorporation of biotin specifically into structures containing a terminal galactose residue (Haselbeck & Hösel, 1993). On establishing this method it became clear that the semi-purified pilin preparations contained a non-specific protein band (possibly a biotin carboxyl carrier protein) that was detected in the absence of biotin hydrazide (results not shown). The predicted molecular mass of the biotin carboxyl carrier protein in N. meningitidis strain Z2491, based on the preliminary Sanger genome sequence data, is 15.9 kDa, which is consistent with its migration in SDS-PAGE. The presence of this non-specific band is evidently due to the co-isolation of this protein in the semi-purified pilin preparations. To demonstrate that this non-specific band did not co-migrate with pilin, and therefore that pilin could be detected by the galactose-specific method, a split-band experiment was carried out (Fig. 3). In this experiment, following separation of pilin by SDS-PAGE, each lane was cut vertically. Pilin was then detected using SM1 and the non-specific band was detected with biotin/streptavidin. In all cases the non-specific band did not co-migrate with pilin. The increased concentration of the non-specific band in the pgIB and pgIC mutant pilin preparations resulted from a higher proportion being co-purified due to a smaller difference between the molecular mass of pilin from these mutant strains and the non-specific band.

Having established that the non-specific band was not pilin and did not co-migrate with any of the pilin samples, we then used this method to detect pilin isolated from C311#3 and its derivatives. The wild-type pilin of C311#3 was detected using this method but not the pilin isolated from the pgIB, pgIC, pgID, pgLA or galE mutants (Fig. 4c). The specificity of the detection method was shown by the lack of reactivity of C311#3 pilin in the absence of galactose oxidase (Fig. 4b). The detection of isolated pilin with the monoclonal antibody SM1 (Fig. 4a) and the lack of detection with the terminal galactose-specific method strongly suggests that the pilin produced by the pgLA, pgIB, pgIC and pgID mutants of C311#3 (as well as its galE mutant, which is unable to synthesize UDP-galactose) do not contain a terminal galactose residue.

Antiserum specific for the C311#3 trisaccharide does not react with pilin isolated from the C311#3 pgLA, pgLB, pgIC, pgID or galE mutants. The specificity of anti-trisaccharide serum was confirmed in Western blot experiments in which it bound pilin isolated from C311#3 but not pilin isolated from C311#3pgLA or C311#3galE (see Methods and Fig. 5b). Nucleotide sequencing experiments have shown previously that the amino acid sequences of the pilin expressed by C311#3 and C311#3galE are identical except for the presence of the complete trisaccharide structure on C311#3 (see Fig. 8), indicating that the anti-trisaccharide serum specifically recognizes the C311#3 trisaccharide. Similarly, anti-pgLA serum was shown to bind pilin isolated from C311#3pgLA and C311#3galE but not pilin isolated from C311#3 (see Methods and Fig. 5c), indicating that anti-pgL is specific for the truncated glycosyl structures of C311#3pgLA and C311#3galE (Fig. 8).

Western blots of semi-purified pilin from C311#3, C311#3pgLA, pgLB, pgIC, pgID and galE mutants were probed with SM1 (Fig. 5a), anti-trisaccharide serum and
anti-\(pglA\) serum. Anti-trisaccharide serum reacted with C311\#3 pilin but not with pilin isolated from the C311\#3 \(pglA\), \(pglB\), \(pglC\), \(pglD\) or \(galE\) mutants (Fig. 5b), indicating that the complete pilin-linked trisaccharide structure was only present in the wild-type. Anti-\(pglA\) serum reacted with C311\#3\(pglA\) and C311\#3\(galE\) pilin but not with wild-type pilin nor pilin isolated from any of the C311\#3 \(pglB\), \(pglC\) or \(pglD\) mutants (Fig. 5c). This confirmed in each case that pilin from the \(pglB\) mutant was not modified either by the complete pilin-linked trisaccharide structure or by a truncated form such as is present in the C311\#3\(pglA\) and C311\#3\(galE\) mutants.

Electron micrographs of the pili produced by the \(pglB\), \(pglC\) and \(pglD::kan\) mutants show no effect on pilus morphology. To determine whether the \(pglB\), \(pglC\) and \(pglD::kan\) mutant derivatives of strain C311\#3 still expressed pili located on the cell surface, and whether these pili had altered morphology, the pili of the \(pglB\), \(pglC\) and \(pglD::kan\) mutants were observed by electron microscopy. All mutant derivatives were found to surface-express pili. A large degree of variation (within samples) in both piliation level and presence or absence of bundles was encountered. In general, there were no consistent differences in pilus morphology or piliation level of the \(pglB\), \(pglC\) and \(pglD::kan\) mutants in comparison to wild-type. A representative electron micrograph of the pili of the \(pglB\) mutant compared to C311\#3 is shown in Fig. 6.

**Distribution of the \(pglB\), \(pglC\), \(pglD\) and avtA genes amongst different \(N. meningitidis\) strains and other species of Neisseria**

To determine the potential of other \(N. meningitidis\) strains and \(Neisseria\) species to make glycosylated pilin structures, a collection of strains and species was surveyed using the probes described in the legend of Fig. 1. The resulting Southern blot (Fig. 7a) clearly demonstrates the presence of \(pglB\), \(pglC\), \(pglD\) and \(avtA\) in all of the \(N. meningitidis\) strains tested. Furthermore, Southern blots performed using single isolates of pathogenic and non-pathogenic \(Neisseria \) spp. revealed that sequences homologous to \(pglB\), \(pglC\), \(pglD\) and \(avtA\) were present in \(N. subflava\), \(N. gonorrhoeae\) and \(N. lactamica\) L19 (Fig. 7b). In addition, \(N. cinerea\), \(N. pharyngis\) and \(N. lactamica\) L12 had homologues to either two or three of the four genes.

**DISCUSSION**

Glycosylation of prokaryotic proteins has been considered to be highly unusual. There are now, however, an increasing number of reports not only of prokaryotic protein glycosylation but also of the importance of glycoproteins in pathogenicity (reviewed by Tuomanen, 1996). To date, the best-characterized prokaryotic glycoprotein is the pilin of \(N. meningitidis\). The structure of the \(N. meningitidis\) strain C311\#3 pilin-linked trisaccharide has been elucidated by carbohydrate analysis and mass spectrometry of tryptic peptides (reviewed by Virji, 1997; see Fig. 8). Marceau et al. (1998) have alternatively identified a disaccharide modification on \(N. meningitidis\). In a recent paper Jennings et al. (1998) described the first gene involved in the glycosylation of pilin. The gene identified, \(pglA\), was found to encode a pilin-specific glycosyltransferase involved in the addition of galactose to the trisaccharide.

In this study we have characterized a genetic locus, \(pglBCD\), from \(N. meningitidis\) that appears to encode additional enzymes involved in pilin glycosylation.
confirmed the absence of the complete pilin-linked trisaccharide from the pilin of each of the pglB, pglC and pglD mutants using antisera specific for either the complete trisaccharide or a truncated form.

The structure of the pilin-linked trisaccharide of C311#3 is shown in Fig. 8. To make this structure, two classes of enzyme are required: glycosyltransferases and enzymes involved in the biosynthesis of the unusual 2,4-dideoxyhexose. Glycosyltransferases are required for the Gal(β1-4)Gal linkage, Gal(α1-3)DATDH linkages and the O-linkage of DATDH to serine 63. In our previous work we have proposed that pglA catalyses the Gal(α1-3)DATDH linkage; therefore two further transferases remain to be identified. Galactose is not specific to the pilin-linked trisaccharide structure, also being present in LPS, but the DATDH residue is pilin-specific.

Deoxy and dideoxy sugars, in particular 2,6- and 4,6-dideoxyhexoses, are found as components of glycoproteins, bacterial cell walls, and numerous secondary metabolites (Thorsen et al., 1993). However, acetamido sugars are unusual. The biosynthesis of a 4-acetamido-4,6-dideoxyhexose in E. coli has been described by Dietzler & Strominger (1973) and Matsuhashi & Strominger (1964). The biosynthesis of this sugar first requires a dehydratase step at the carbon position C4 of the hexose residue, followed by transamination and acetylation. The stereospecificity of the N. meningitidis DATDH residue, and therefore its likely precursor molecule, is unknown. However, we predict that the biosynthesis of this sugar will follow the pathway described (above) for acetamido sugars. Therefore, the biosynthesis of this residue would require several specific enzyme activities, including dehydratases, transaminases and acetyltransferases. Homologues found to the pglB, pglC and pglD genes suggest they have roles in the biosynthesis of this amino sugar residue.

The first ORF identified in the locus, pglB, possesses two regions of distinct homology which also have distinct G+C contents. This suggests a recent in-frame fusion of two ORFs resulting in a bifunctional protein. For this reason, the two distinct regions will be discussed separately. The amino-terminal region of the putative

Fig. 7. Distribution of pglA, pglB, pglC and avtA in various N. meningitidis strains and Neisseria species. The panels show the results of Southern hybridization experiments with probes specific for the pglB, pglC, pglD and avtA genes (See Methods). Chromosomal DNA (0.2–1.0 µg) from a range of strains was digested with ClaI and separated on a 1% agarose gel. The arrowheads on the left of the figure indicate the positions of molecular mass markers (8 kbp and 5 kbp in ascending order). Slight variations in band intensity are in direct proportion to the amount of DNA in each lane as judged by ethidium bromide staining. The N. meningitidis strains (a) and Neisseria species (b) used are indicated at the top of the figure.

Fig. 8. Proposed structure of the pilin-linked trisaccharide of C311#3 and its derivatives (see Stimson et al., 1995). The arrow indicates the proposed truncation of the trisaccharide structure in galE and pglA mutant strains. The bracket at the top indicates the DATDH residue; the genes proposed to be involved in biosynthesis of this residue are indicated above.
PglB protein is highly homologous with a family of putative glycosyltransferases. These glycosyltransferases are proposed to transfer a sugar residue (usually galactose) to the lipid precursor undecaprenol phosphate (Wood et al., 1999; Fry et al., 1998; Drummelsmith & Whitfield, 1999). This region of PglB also contains the hydrophobic domain that is required by these proteins for interaction with the undecaprenol phosphate lipid carrier (Wood et al., 1999). The transfer of a nucleotide sugar to a lipid carrier is often the first step in oligosaccharide biosynthesis. It is therefore suggested that PglB may be required in the transfer of the first sugar residue either to an undecaprenol phosphate lipid carrier or directly to the serine 63 of pilin. This transfer may represent the initial step in the glycosylation of pilin.

The carboxy-terminal 168 aa stretch of the putative PglB protein shows strong homology with a family of acetyltransferases (e.g. UDP-N-acetylglucosamine and sialic acid transfer) required for LPS and capsular polysaccharide biosynthesis (Awram & Smit, 1998; Fry et al., 1998; Annunziato et al., 1995). Typical of this family of acetyltransferases, PglB contains an isoleucine patch which is a common signature associated with acetyltransferase function (Dicker & Seetharam, 1992). The biosynthesis of acetamido sugars such as the 2,4-diactetamido sugar residue found in the pilin-linked trisaccharide requires acetylation of the amino groups. Therefore, this region of PglB is suggested to be involved in the acetylation at C2 or C4 of the diactetamido sugar constituent of the pilin-linked trisaccharide.

Thus it appears that PglB may be a bifunctional protein involved in the early steps of trisaccharide biosynthesis, namely in the biosynthesis of the first sugar residue and its transfer either to an undecaprenol phosphate lipid carrier or to serine 63 of the pilin. The pilin produced by the pglB::kan mutant would be expected to be devoid of the complete trisaccharide structure. The altered migration, the absence of a terminal galactose residue and the failure of pilin isolated from the pglB::kan mutant to react with either anti-trisaccharide or anti-pglA sera support this hypothesis.

The second ORF identified in the locus, pglC, shows significant homology with a family of aminotransferases involved in the synthesis of amino sugars (Stroehler et al., 1995). The putative PglC protein is strongly similar to RfbE of E. coli, which is a probable perosamine synthetase. RfbE is believed to catalyse the transamination of GDP-4-keto-6-deoxyxamnnon to GDP-4-amino-4,6-dideoxyxamnnon (perosamine; Stroehler et al., 1995). PglC is also highly similar to NikC from Streptomyces Tu901, which has been shown to encode an L-lysine 2-aminotransferase required for the biosynthesis of nikkomycin (Bruntner & Bormann, 1998). All of these proteins belong to a novel class of pyridoxamine- or pyridoxal-phosphate-dependent dehydrogenases and aminotransferases, based on the presence of a pyridoxal-phosphate-binding motif (PS00600; Appel et al., 1994). This is consistent with the findings of Matsushashi & Strominger (1966), where a pyridoxal-phosphate-dependent transaminase is required for the biosynthesis of an acetamido sugar. This leads us to propose that in N. meningitidis PglC is required for the transamination of either C2 or C4. Therefore, we would expect the pglC::kan mutant to be unable to synthesize a complete DATDH residue, resulting in pilin with either a single sugar residue or no glycosylation depending on the substrate specificity of the glycosyltransferases. The possibility of complete absence of glycosylation on the serine 63 of pilin isolated from the pglC::kan mutant is supported by its increased migration, its failure to be detected by a terminal galactose-specific stain and its failure to be detected by either anti-trisaccharide or anti-pglA sera.

The pglD gene product is highly similar to proteins essential for either LPS or O-antigen amino sugar biosynthesis (Skurnik et al., 1995; Comstock et al., 1996). These proteins all have maximum homology in their carboxy-termini and are divergent in their amino-termini. Fallarino et al. (1997) suggested that this was due to the amino-terminus being involved in specificity and the carboxy-terminus incorporating the functional domain of these proteins. The putative PglD protein shares these characteristics and also contains the two distinct motifs, both for binding NAD, that have been suggested to be a requirement for enzyme function (Skurnik et al., 1995). PglD demonstrated high similarity to the TrsG from Y. enterocolitica, which is proposed to be an epimerase or dehydratase involved in N-acetyl-galactosamine biosynthesis (Skurnik et al., 1995). The BplL protein of Bordetella pertussis is also highly similar to PglD and itself has some strong similarities to a number of dTDP-glucose 4,6-dehydrogenases (Bechthold et al., 1995; Linton et al., 1995). Based on this observation, Allen & Maskell (1996) suggest that BplL is responsible for the biosynthesis of FucNAcMe (a 6-deoxy derivative of galactose). They further suggest that this group of enzymes are generally required for the synthesis of 6-deoxy and dideoxy sugars.

It seems likely, therefore, that PglD is involved in synthesis of the DATDH moiety of the pilin-linked trisaccharide as a dehydratase acting on C2, C4 or C6. Therefore, we would expect a similar modification (i.e. one or no sugar residues) to be present on the pglD::kan mutant pilin, as is expected for the pglC::kan mutant. This hypothesis is again supported both by the decreased migration of pilin isolated from the mutant compared to the wild-type, and also by the failure of this pilin to be detected by a terminal galactose-specific method, anti-trisaccharide or anti-pglA sera.

The final ORF in the identified locus, AvtA, has a high degree of similarity to putative alanine-to-valine aminotransferases (e.g. AvtA of E. coli; Sofia et al., 1994). These genes are involved in amino acid biosynthesis and have not been implicated in sugar biosynthesis. Several attempts were made to knock out the avtA gene of N. meningitidis, but they were unsuccessful, indicating a possible essential housekeeping function for this protein. Therefore, the role (if any) of this gene in pilin glycosylation could not be determined.
Table 3. Comparison of the neisserial pilin glycosylation genes with a flagellin glycosylation locus identified in Campylobacter jejuni

<table>
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<tr>
<th>C. jejuni gene</th>
<th>Neisserial pilin glycosylation gene:</th>
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<tr>
<td>pgIA</td>
<td>pgIB&lt;sup&gt;a&lt;/sup&gt; pgIC pgID</td>
</tr>
<tr>
<td>pgIA</td>
<td>30/49</td>
</tr>
<tr>
<td>pgIC</td>
<td>51/64</td>
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<tr>
<td>pgID</td>
<td>32/56</td>
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<tr>
<td>pgIE</td>
<td>27/43</td>
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<tr>
<td>pgIF</td>
<td>33/49</td>
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<sup>a</sup> Homology to C. jejuni pgIC and pgIF is in the amino-terminal and carboxy-terminal region, respectively (see text).

The functions proposed for the pgIB, pgIC and pgID genes, and their close proximity to each other, suggest that they act together in the biosynthesis of the acetamido sugar residue of the pilin-linked trisaccharide. The biosynthesis of an acetamido sugar, as described by Matsushima & Strominger (1966), is initiated by dehydration (PgID dependent), followed by transamination (PgIC dependent) and transacetylation (PgIB dependent) of a single carbon. This acetamido sugar could then be transferred to either a lipid carrier or a C4 acetamido sugar (for example glucosamine or fucosamine).

A survey to detect the presence of pgIB, pgIC, pgID and avtA, using Southern analysis, revealed that these genes were present in all of the N. meningitidis strains tested, in N. gonorrhoeae and also in other species of Neisseria. This was also found with the previously identified pgIA gene, suggesting that pilin glycosylation may be common amongst neisserial species (Jennings et al., 1998). During the preparation of this paper, Szymanski et al. (1999) reported the identification of a Campylobacter jejuni locus involved in the biosynthesis of an undefined glycosylation on the flagella of this organism. Although the sequence identities were not amongst the highest matches found, the genes were present in the same order as the pgIBCD locus described here (see Table 3). Szymanski et al. (1999) not only implicated the locus in glycosylation of flagella, but also of other proteins, confirming that their locus represents a general glycosylation pathway.

In conclusion, this study has identified three genes, pgIB, pgIC and pgID, involved in pilin glycosylation, in addition to the previously identified pgIA gene (Jennings et al., 1998). To date, the precise functions that we have predicted for pgIB, pgIC and pgID are based on amino acid sequence homologies with genes in the databases that are involved in the biosynthesis of similar chemical structures to the pilin-linked trisaccharide. Currently we are obtaining structural data and biochemical evidence to support the gene functions that we have proposed.

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