The group I pilin glycan affects type IVa pilus hydrophobicity and twitching motility in *Pseudomonas aeruginosa* 1244

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The group I pilin category is the most common type of type IVa pilus produced by *Pseudomonas aeruginosa*. The lateral surfaces of these pili are characterized by the presence of closely spaced, covalently attached O-antigen repeating units. The current work was conducted to investigate the pilin glycan’s effect on pilus solubility and function. Culture supernatant fluids containing fully, partially and non-glycosylated *P. aeruginosa* group I pili were tested for solubility in the presence of ammonium sulfate. These results showed that while pili expressing three or four sugars were highly soluble under all conditions, those with fewer than three were insoluble under the lowest salt concentrations tested. A representative of the *P. aeruginosa* group II pili also showed low solubility when assayed under these same conditions. Reduced solubility suggested an increased pilus surface hydrophobicity, which was supported by protein modeling. While having no effect on the WT strain, an ionic strength found at many host infection sites inhibited surface and subsurface twitching motility of strain 1244G7, an isogenic mutant unable to glycosylate pilin. This effect was reversed by mutant complementation. Twitching motility of *P. aeruginosa* strain PA103, which produces group II pili, was also inhibited by ionic strengths which influenced the mutant 1244 strain. We suggest that the group I pilin glycan may, therefore, be beneficial to this organism specifically for optimal pilus functioning at the many host disease sites with ionic strengths comparable to those tested here.

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The type IVa pilus is a protein fibre that extends from the cell surface. This structure, which is composed of a monomeric subunit called pilin, is an important virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa*, where it has long been recognized as an adhesion factor (Hahn, 1997) as well as being responsible for a type of surface motility called twitching (Burrows, 2012). The type IVa pili of *P. aeruginosa* can be placed into one of five groupings (I through V) based on pilin primary structure and the presence of pilus-specific accessory genes (Castric, 1995; Castric & Deal, 1994; Kus et al., 2004), although the majority of isolates reported produce pilin belonging to groups I and II (Kus et al., 2004). The *P. aeruginosa* group I pilins are further characterized by the presence of a covalently attached O-antigen repeating unit (Castric et al., 2001). The addition of this structure is catalysed by an oligosaccharyltransferase called PilO (aka TpfO), which is encoded by the accessory gene pilO located directly downstream from the pilA gene (Castric, 1995; Castric et al., 2001). It is interesting to note that *P. aeruginosa* group IV pilins are also glycosylated, although the glycan substrate employed in this situation is not the O-antigen repeating unit (Harvey et al., 2011; Voisin et al., 2007).

While a number of studies have shown that the type IVa pilus tip is important for adhesion to biotic and abiotic surfaces (Farinha et al., 1994; Fletcher et al., 1993; Giltner et al., 2006; Woods et al., 1980), the influence of the lateral surface of this fibre on pilus function has not been examined. The number of pili produced per cell as well as pilus length suggests the possibility of significant contact between the fibre surface and the pathogen’s physical environment. Such an interaction could affect the pathogenicity of this organism by influencing a number of surface-associated virulence factors including twitching motility, biofilm development, and contact with innate defence and target host cell surfaces.

The type IVa pilus exterior, as determined by protein features alone, is a mosaic of repeated pilin surface structures displaying identical patterns composed of ionized charges, weak bonds and hydrophobic regions (Craig et al., 2004).
attraction of these forces between fibres lends itself to bundle formation (Brinton, 1965; Silipigni-Fusco, 1987) which could be detrimental to pilus extension and retraction, a process necessary for most pilus functions (Burrows, 2012). The *P. aeruginosa* 1244 pilin glycan is covalently attached through its reducing end to the β-hydroxyl carbon of the serine which resides at the pilin C terminus (Castric *et al.*, 2001; Comer *et al.*, 2002). This structure is evenly distributed over the pilus surface (Smedley *et al.*, 2005) and so would be expected to have a significant effect on the physical properties of these fibres by contributing charge and hydrophilicity as well as by shielding the surface amino acid residues from the fibre environment. These effects would be expected to reduce the pilus–pilus attraction described above, thereby positively influencing functionality. The glycan modification of the *P. aeruginosa* type IVa pilus–pilus attraction would be expected to reduce the physical properties of these fibres by contributing charge and hydrophilicity as well as by shielding the surface amino acid residues from the fibre environment.

The work described in the present study suggests that the pilin glycan strongly influences pilus–pilus interactions. Evidence is presented that the enhanced solubility of the pilin glycan affects pilus function. An isogenic mutant of strain 1244 that lacked the ability to glycosylate pilin demonstrated reduced twitching motility, a trait important for virulence of this organism (Silipigni-Fusco, 1987) broth cultures were grown at 37 °C on a rotary shaker at 250 r.p.m. Solid media were prepared with the addition of either 1.5 % (LB) or 2.0 % agar. When necessary, media were supplemented with carbenicillin at 200 μg ml⁻¹ or gentamicin (Gm) 250 μg ml⁻¹ for *P. aeruginosa* and 10 μg ml⁻¹ for *Escherichia coli*. Kanamycin was used at a concentration of 35 μg ml⁻¹. For induction of pPAC46, media were supplemented with 5 mM IPTG. See Table 1 for all strains and vectors used.

**Table 1.** Strains and vectors used in this study

<table>
<thead>
<tr>
<th>Strains/vector</th>
<th>Description*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1244</td>
<td>WT, group I pili</td>
<td>Ramphal <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>1244G7</td>
<td>PilO-negative mutant, Gm'</td>
<td>Smedley <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>1244A7</td>
<td>PilA-negative mutant, Hg'</td>
<td>Horzempa <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>1244.2.1</td>
<td>Transposon mutant, incomplete pilin glycosylation, Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>PA103</td>
<td>WT, group II pili</td>
<td>Liu (1973)</td>
</tr>
<tr>
<td>PA103ApilA</td>
<td>PilA-negative mutant, Gm', 577B</td>
<td>Comolli <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>A031</td>
<td>Clinical isolate, group I pili, PilO-negative.</td>
<td>Castric &amp; Deal (1994)</td>
</tr>
<tr>
<td>pPAC24</td>
<td>pMMB66EH with 1244 pilA</td>
<td>Castric (1995)</td>
</tr>
<tr>
<td>pPAC46</td>
<td>pMMB66EH with 1244 pilAO</td>
<td>Castric (1995)</td>
</tr>
<tr>
<td>pMBT100</td>
<td>pMMB66EH with 1244 pilO</td>
<td>Smidley <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>pTJ5S3</td>
<td>pUC8 containing RK2 oriT</td>
<td>Schmidhauser &amp; Helinski (1985)</td>
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<tr>
<td>pPC110</td>
<td>pUC7 containing a Gm' cassette</td>
<td>Nunn <em>et al.</em> (1990)</td>
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<td>pSMC900</td>
<td>pTJ5S3, Tn5, Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>λ467</td>
<td>Tn5 vector, Km'</td>
<td>Yelton <em>et al.</em> (1983)</td>
</tr>
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</table>

*Gm', gentamicin resistance; Hg', mercury resistance; Ap', carbenicillin resistance; Km', kanamycin resistance.

METHODS

**Bacterial culture conditions.** Luria–Bertani medium (LB; 1 % tryptone, 0.5 % yeast extract, 1 % NaCl, pH 7.0) or Casamino acids–yeast extract medium (CAYE; 0.75 % Casamino acids, 0.15 % yeast extract) (Silipigni-Fusco, 1987) broth cultures were grown at 37 °C on a rotary shaker at 250 r.p.m. Solid media were prepared with the addition of either 1.5 % (LB) or 2.0 % agar. When necessary, media were supplemented with carbenicillin at 200 μg ml⁻¹ or gentamicin (Gm) 250 μg ml⁻¹ for *P. aeruginosa* and 10 μg ml⁻¹ for *Escherichia coli*. Kanamycin was used at a concentration of 35 μg ml⁻¹. For induction of pPAC46, media were supplemented with 5 mM IPTG. See Table 1 for all strains and vectors used.
pSMC900 was introduced into \textit{P. aeruginosa} 1244 by triparental mating (Figurski & Helinski, 1979; Ruvkun & Ausubel, 1981). \textit{Gm} \textsuperscript{+} colonies were patched to LB-\textit{Gm} plates which were incubated overnight at 37°C. Colonies were lifted using nitrocellulose membranes, which were blocked and then probed with monoclonal 11.14 (Castric \textit{et al.}, 2001), an antibody which is specific for strain 1244 O-antigen/pilin glycan. Forty-four colonies of 1771 screened were found to be non-reactive. On re-examination, two of these clones did not react with monoclonal 11.14, but were recognized by a pilin-specific monoclonal antibody (results not shown). One of these strains, 1244.2.1, was used in the present study. Pili produced by this strain were subjected to Western blot analysis, using a protocol described by Castric (1995) and employing a 1244-pilin-specific monoclonal antibody 5.44 (Castric & Deal, 1994) as probe. Results indicated that this strain produced pilin of a reduced apparent molecular mass as compared with the WT (Fig. 1a). The reduced electrophoretic mobility of strain 1244G7 pilin was previously shown to be due to the absence of the O-antigen repeating unit (Smedley \textit{et al.}, 2005). To examine this point further, purified pilin samples of strains 1244 and 1244.2.1 separated by SDS-PAGE were submitted to The University of Pittsburgh Genomics and Proteomics Core Laboratory for chymotrypsin digestion followed by mass spectrometric analysis using high resolution LC-MS/MS (LTQ/Orbitrap). This treatment revealed a fragment with the expected C-terminal sequence ([KPNYAPANCPKS]) and a mass of 1608.61 kDa. This is consistent with the presence of the peptide plus FucNac and xylose, but the loss of pseudamidic acid. A similar analysis of strain 1244 pilin revealed a fragment with this same sequence, but a mass of 1954.89 kDa, which is consistent with the presence of the intact glycan.

\textit{O}-Glc-\{\beta\rightarrow3\}\textit{a}-FucNac-(\textit{z}\rightarrow3)\textit{D}-FucNac-(\beta\rightarrow). This O-antigen repeating unit is produced by \textit{P. aeruginosa} PA103 (Goldberg \textit{et al.}, 1992). Western blot analysis, as described above, of mutant PA103Δ\textit{pilA} expressing a functional 1244 \textit{pilA} gene in the absence of \textit{pilO} produced non-glycosylated pilin as indicated by electrophoretic mobility (Fig. 1b). An increased apparent molecular mass in the presence of both of these genes indicated the production of only glycosylated pilin.

\textit{L-Rha-}\{\textit{z}\rightarrow3\}\textit{L}-FucNac-(\textit{z}\rightarrow3)\textit{L}-FucNac-(\textit{z}\rightarrow3)\textit{D}-QuiNac-(\textit{z}\rightarrow). \textit{P. aeruginosa} strain 577B (Castric & Deal, 1994) produces group I pilin and belongs to International Antigenic Typing System (IATS) serotype O4. The O-antigen repeating unit of this type is the four-sugar structure listed here (Knirel \textit{et al.}, 2006). Pilin from \textit{P. aeruginosa} A031, which is composed of pilin identical in primary structure to that from strain 577B (T. M. Allison & P. Castric, unpublished data) was used as a control. Pilin monomers from this strain are not glycosylated as determined by Western blot (Fig. 1c).

**Pilus preparation.** Four 2 % CAYE agar plates for each \textit{P. aeruginosa} strain to be tested were inoculated with CAYE broth starter cultures and incubated for 14 h at 37°C. These cells were resuspended with CAYE broth and diluted to an equal OD650 using the same medium. This suspension was sheared by passage through a 19-gauge needle, stirred for 30 min at room temperature, vortexed for 45 s, and centrifuged at 11 952 \textit{g} and 4°C for 30 min. The cell pellets were discarded and the supernatant fractions were transferred to 15 ml glass round-bottom centrifuge tubes.

**Pilus solubility assays.** Protein from each culture supernatant was harvested in three sequential salt precipitations. Water saturated with ammonium sulfate was first added to each initial culture supernatant sample to a salt concentration of 0.1 M. This suspension was incubated on ice for 30 min then centrifuged for 30 min at 11 952 \textit{g} at 4°C. Next, solid ammonium sulfate was added to each of the separated supernatant fluids produced by the previous step to a concentration of 1.11 M. This was incubated and centrifuged as above. The previous step was repeated on the resulting supernatant fluid using solid ammonium sulfate added to a final concentration of 3.95 M.

The pellets from each of these samples were dissolved in deionized water and subjected to (SDS) PAGE using a 16 % T separation gel as previously described (Castric \textit{et al.}, 1989). All protein samples were diluted so as to produce pilin levels that fit within the linear portion of a previously determined pilin protein calibration curve (Furst, 2005). Proteins were then electroblotted onto 0.45 μm nitrocellulose membranes at 100 V for 20 min using a Bio-Rad Mini Trans-blot system. This treatment ensured that the pilin sample left the gel but did not pass through the membrane (Furst, 2005). The membranes were blocked in protein solution (Sidberry \textit{et al.}, 1985) for 20 min and incubated overnight with either monoclonal antibody 5.44 (Castric \textit{et al.}, 2001), which is specific for group I pilin, or monoclonal antibody 2.97 (Saiman \textit{et al.}, 1989), which recognizes \textit{P. aeruginosa} PA103 pilin. The membranes were washed with PBS three times for 10 min, then incubated with a goat anti-mouse Alexa Fluor 532 labelled 

![Fig. 1. Western blot of \textit{P. aeruginosa} whole-cell extracts demonstrating the influence of the pilin glycan on protein electrophoretic mobility. (a) Strain 1244.2.1 pilin. The reduced migration of strain 1244G7 pilin was previously shown to be due to the absence of the O-antigen repeating unit (Smedley \textit{et al.}, 2005). (b) Pilin produced by PA103Δ\textit{pilA}/\textit{pPAC46}. The presence of \textit{pilO} results in glycosylation of 1244 pilin. (c) Strain 577B and A031 pilins. These pilins have identical primary structures. All analyses employed a group I pilin-specific monoclonal antibody 5.44 (Castric & Deal, 1994) as probe.](image-url)
secondary antibody for 1 h with constant motion in darkness. The membranes were washed again as above in darkness and allowed to dry. Fluorescent protein bands were visualized using a FluorImager 595 (Molecular Dynamics) at 514 nm with a 570 DF30 filter. The fluorescence was then quantified using ImageQuant version 5.0 software (Molecular Dynamics).

**Twitching assays.** Surface twitching (Semmler et al., 1999) was measured as follows. Cells were grown in CAYE broth to an OD650 of 0.200 (this value gave 4 × 10^7 c.f.u. ml^-1 ). Dilutions calculated to produce 400, 40 and 4 c.f.u. ml^-1 were spread onto 2% CAYE agar with and without 90.0 mM ammonium sulfate. Plates were incubated at 37°C for 20–26 h. Isolated colonies were imaged using a Leica S6D (Leica Microsystems) microscope with Spot Insight Colour camera (Diagnostics Instruments). Multiple fields of view were observed to obtain representative colony morphologies.

Subsurface twitch assays (McMichael, 1992) were conducted as previously described (Castric et al., 2001). Fisherbrand 100 × 15 mm polystyrene plates were used for the standard subsurface assay in which case twitch zones were measured after 48 h of incubation. BD Falcon 100 × 20 mm vacuum gas plasma treated polystyrene tissue-culture dishes were used in all other assays. Here, incubation time was 24 h. At least 40 repeats of each treatment were carried out.

**Statistical analysis.** Statistical analyses for the subsurface twitching assays of 1244 and 1244G7 at increasing concentrations of ammonium sulfate were conducted using SPSS statistical software for one-way ANOVA followed by Bonferroni correction. Paired t-tests were conducted for all pairwise comparisons of subsurface twitching using the online Graphpad QuickCalcul software.

**Pilin surface models.** Pilin sequences for 1244 (group I) and PA103 (group II) were submitted to the PHYRE 2 online protein structure prediction tool (Kelley & Sternberg, 2009). Surface analysis and modelling was conducted using DS ViewerPro 6.0 software.

## RESULTS

### Influence of glycan components on pilus–pilus interaction

The *P. aeruginosa* 1244 pilin oligosaccharide (Fig. 2a) is composed of N-acetyl fucosamine, xylose and pseudaminic acid (Castric et al., 2001). This structure is attached, via the FucNAc reducing end, to the \( \beta \)-carbon of the serine that resides at the pilin C terminus (Comer et al., 2002). Solubility is an appropriate way to determine pilus–pilus interaction via the fibre lateral surface since it is known that salt precipitation produces paracrystalline structure formation by longitudinal alignment of these fibres (Brinton, 1965).

In order to establish the influence of the components of this structure on pilus solubility, fully, partially and non-glycosylated group I pilus constructs and strains were prepared and tested. Solubility was determined by ammonium sulfate precipitation from sheared cell supernatant fluids followed by quantitative Western blot of the pellet. The pH of these supernatant fluids was approximately pH 7.9. At this pH, all of the pilus tested are predicted from their primary structures to have a net negative charge.

Fig. 2(a) shows that the presence of the pilin glycan had a dramatic influence on strain 1244 pilus solubility. While the presence of the native oligosaccharide allowed solubility in both 0.1 and 1.11 M ammonium sulfate, the absence of this glycan or the presence of only xylose-FucNAc rendered the fibres insoluble at the lowest salt concentration. Quantification of these blots by fluorometric imaging confirmed these results (Fig. 2b). In order to determine whether the pseudaminic acid residue influenced fibre solubility through the presence of its negative charge or because of the increased oligosaccharide size, pilin from strain 1244 containing three neutral sugars (Glc–FucNAc–FucNAc) were tested. Fig. 2(a) indicates that this pilus form was soluble in 0.1 M ammonium sulfate, suggesting

![Fig. 2](image-url)
that pilus solubility did not require a negative charge but was rather influenced by the length of the glycan. These findings were supported by the evidence that pili (isolated from *P. aeruginosa* 577B), composed of pilin which showed a 95 % identity in primary structure with that from 1244 but which had a four-sugar neutral saccharide (Rha-FucNAc-FucNAc-QuinA), precipitated in the presence of 3.95 M ammonium sulfate, but not at the lower concentrations. Pili for strain A031, which were made up of pilin identical in sequence with that of strain 577B, but which lacked a glycan, were insoluble in 0.1 M ammonium sulfate (Fig. 2a). Again, quantification of these blots by fluorescent imaging confirmed these results (Fig. 2b). For comparison, the solubility of a naturally non-glycosylated type IVa pilus of *P. aeruginosa* was carried out. Here, it was found that the pili of strain PA103, which belonged to group II, were insoluble in 0.1 M ammonium sulfate (Fig. 2a, b).

Overall, these results suggest that pilus solubility is enhanced by the length of the glycan rather than the presence of the negative charge. Such a clear effect on the interaction between the pili and its local environment suggests the possibility that the presence of this oligosaccharide has an influence on pilus function.

**Predicted pilin hydrophobicity**

The reduction of the *P. aeruginosa* 1244 pilus solubility in the presence of elevated salts suggests that the protein portion of this pilus surface contains hydrophobic components. While the native structure of the *P. aeruginosa* strain 1244 pilus is not known, modelling can be used to produce a representation of the fibre surface based on the knowledge that the β-sheet region of the subunit resides at the polymer exterior of type IVa pili (Craig *et al.*, 2004). Fig. 3 presents models for the group I pilin from strain 1244 and the group II pilus subunit of strain PA103. Here it can be seen that residues containing ionizable R-groups are evident as are amino acids (serine and threonine) capable of hydrogen-bond interaction. Of particular interest are the distinctive hydrophobic areas. These residues, when arranged in the pilus helix, would form a repeating pattern of surface hydrophobicity that could potentially influence interaction with the cell’s environment. The proximity of the O-antigen repeating unit attachment site to this area (Fig. 3a, red star) suggests that this oligosaccharide has the potential to moderate the influence of the hydrophobic region. The strain PA103 pilus surface also has a hydrophobic region which may account for the reduced solubility seen above (Fig. 2a, b).

**Influence of pilus glycan on twitching motility**

Twitching motility on the surface of agar plates is associated with the presence of flared colony edges (Henrichsen *et al.*, 1972). This phenomenon was observed with growth of either strain 1244 or strain 1244G7 (which produces only non-glycosylated pilin) on CAYE agar in the absence of added ammonium sulfate (Fig. 4a, b). Flared colony edges were also seen with the WT strain growing in the presence of 90 mM ammonium sulfate (Fig. 4e) while strain 1244G7 growing on the same medium presented smooth-edged colonies (Fig. 4f) suggesting an inhibition of pilus-mediated motility. Strain 1244.47, an isogenic pilA mutant (Horzempa *et al.*, 2006), growing on this same medium confirmed the relationship between this colony morphology and the presence of functional pili (Fig. 4c); strain 1244.47 does not show an altered morphology when growing in the presence of elevated ammonium sulfate (Fig. 4g). Assay of culture supernatant fluids by quantitative Western blot indicated that strain 1244G7 produced pili at the same level as the WT (T. M. Allison & P. Castric, unpublished data) suggesting that twitching reduction was not due to inhibition of piliation. Both strains carried out a normal twitching response when sucrose was substituted for ammonium.
sulfate in a concentration of 250 mM (T. M. Allison & P. Castric, unpublished data), which indicates that the response seen was not due to an osmotic effect. In order to determine whether the salt effect on colonial morphology was due to pilin glycosylation, strain 1244G7 containing either an empty vector or the \( \textit{pilO} \) gene under control of a \( \textit{tac} \) promoter was tested. As can be seen in Fig. 4(d, h), expression of this gene partially restores the wild-type colonial morphology. These results suggested that the colonial morphology change seen was due to a selective salt inhibition of twitching motility of cells producing non-glycosylated pili.

To study this response further, strains 1244 and 1244G7 were tested using the subsurface twitching motility assay. Fig. 5 shows that the mutant producing non-glycosylated pili showed progressively reduced twitching motility with increasing ammonium sulfate concentration. This inhibition became statistically significant \((P<0.0001)\) at 60 mM ammonium sulfate. Additionally, the mutant strain exhibited reduced twitching compared with the WT strain even at the lower salt concentrations (Fig. 5). The WT parent showed no motility inhibition at any salt concentration tested. Analysis of these results (as indicated in the legend of this figure) shows them to be statistically valid. In comparison, inclusion of 250 mM sucrose in the assay plates did not selectively influence twitching motility by either strain (T. M. Allison & P. Castric, unpublished data), suggesting that the selective ammonium sulfate inhibition was not due to an osmotic pressure effect on 1244G7. Selective inhibition of 1244G7 twitching by 125 mM sodium chloride (Fig. 6a) suggested that the effect seen with ammonium sulfate was due to ionic strength and not specifically to ammonium or sulfate ions. The expression of a functional \( \textit{pilO} \) gene, which had previously been shown to complement glycosylation in 1244G7 (Smedley \textit{et al.}, 2005), reversed salt inhibition (Fig. 6a) indicating that selective ammonium sulfate inhibition of twitching motility was not due to a secondary mutation. Fig. 6(b) shows that ammonium sulfate, at a concentration which inhibited 1244G7, also inhibited twitching motility.
of *P. aeruginosa* PA103, a group II strain that produces non-glycosylated pili (DiGiandomenico et al., 2002).

The lower solubility of non-glycosylated strain 1244 pili in the presence of 0.1 M ammonium sulfate as well as estimated surface structure suggested that these fibres had an increased hydrophobicity compared with the glycosylated, WT strain 1244. If this is the case, the reduction in twitching motility might be in part due to the hydrophobic interaction among non-glycosylated 1244 pili or between these pili and the external environment. To test whether the non-glycosylated pili were attracted to the surface of the polystyrene culture plates used in these assays, twitching motility was repeated using plates chemically treated to reduce surface hydrophobicity. Fig. 6(c) shows that, in this situation, there was no difference between glycosylated and non-glycosylated strain 1244 pili under conditions of elevated ammonium sulfate, suggesting that an interaction between the pilus lateral surface and the cell environment is capable of altering pilus function. Incubation times employed using modified test plates were approximately half those of assays used for polystyrene plates. Thus, either 1244 or 1244G7 growing next to the treated surface migrated at approximately four times the rate of the WT traversing the polystyrene plate.

**DISCUSSION**

The presence of the O-antigen repeating units on the exterior of the *P. aeruginosa* group I type IVa pilus have the potential to have a major influence on the physical surface properties of this structure. This oligosaccharide commonly adds a negative charge, while the variation of the O-antigen repeating unit in saccharide structure and number (Knirel, 1990; Lam et al., 2011) would produce a multiplicity of distinctive pilus surfaces. In addition, the glycan structure, unlike the pilin protein surface, has a dynamic conformational potential and would be expected to move freely about the point of attachment. This means that the non-covalent interaction between the glycan and the protein surface from which it extends is difficult to predict because it is likely driven by...
the physical and chemical make-up of the environment in which the cell resides. This degree of variability suggests that the pilin glycan could influence pilus function in a number of ways.

The results of pilus solubility experiments presented in this work suggest that the pilin glycan has an important role in pilus–pilus interaction. These oligosaccharides could influence solubility in several non-exclusive ways. The hydrophilic nature of these structures would be expected to allow them to fit into the water lattice more efficiently than could many of the protein surface residues. In addition, the conformational flexibility of these glycans would minimize the formation of bundles of interacting adjacent fibres. Further, the bulk of the typically three to four sugars present in an O-antigen repeating unit could act to shield the pilus protein surface from direct contact with adjacent fibres or environmental surfaces. The absence of such shielding would lead to an increase in lateral surface attraction such as seen with the type IVb pilus of the enteropathogenic E. coli (Ramboarina et al., 2005) or the paracrystalline structure commonly seen with aggregated type IVa pili (Brinton, 1965). In addition, the O-antigen repeating unit of P. aeruginosa frequently displays a negative charge (Knirel et al., 2006) at physiological pH, which would produce a zone of electrostatic repulsion on the fibre surface. Our results suggest that while electrical charge may enhance pilus function, it is not required for optimal pilus solubility. Rather, sugar number appears to be the key determinant in pilus solubility. However, the loss of solubility protection by fibres containing only two sugars suggests that the O-antigen repeating unit of this organism provides the near minimum protection for fibre solubility.

The decrease in solubility in the absence of optimal glycan number implies the presence of hydrophobic regions on the pilus surface, which was supported by examination of surface-oriented pilin subunits using molecular modelling. Repetition of this hydrophobic region over the length of the pilus could result in a cumulative significant attractive force between adjacent fibres. Previous work (Smedley et al., 2005) indicated that cells of strain 1244G7 had an increased ammonium-sulfate-induced agglutination response as compared with strain 1244. This suggested that the pilin glycan directly influenced cell–cell interaction.

While bundles of fibres increase the success of some bacteria (colonization by enteropathogenic E. coli for example; Humphries et al., 2010), this type of interaction could be detrimental for other organisms. Twitching motility by P. aeruginosa, which is mediated by extension and retraction of the type IVa pilus (Burrows, 2012), is a significant contributor to this organism’s virulence (Comolli et al., 1999; Zolfaghar et al., 2003). Weak binding at multiple pilus lateral sites could have a strong cumulative influence on the efficiency of type IVa pilus extension or retraction processes. The present study, using both the surface and submerged twitch assays, showed that there was little difference between strain 1244 and the isogenic mutant 1244G7 when measured at low ionic strength. However, increasing ionic strength through the addition of ammonium sulfate selectively reduced mobility by the mutant in both types of assay. The higher ionic strengths used in these experiments were in the same range as those found at a variety of P. aeruginosa infection sites including serum (Maas et al., 1985) and the respiratory tract surface fluid (Joris et al., 1993; Knowles et al., 1997; Landry & Eidelman, 2001). This suggests that strains producing group I pilin may have a selective advantage in certain disease situations (Kus et al., 2004). In support of this proposal, previous work has shown that strain 1244G7 had reduced virulence as compared with the WT using a mouse respiratory disease model (Smedley et al., 2005). We show in the present work that twitching motility of P. aeruginosa PA103, a strain which produces a non-glycosylated pilus (DiGiandomenico et al., 2002), is strongly inhibited at elevated ionic strengths. This suggests that strains producing glycosylated group I pilin may have a selective virulence advantage under conditions of elevated ionic strength as compared with group II producers. Further studies will be required to clarify these points. Such studies could suggest that the targeting of the pilin glycosylation reaction is a feasible chemotherapeutic strategy. Since much is known of the PilO substrate requirements, knowledge of the PilO structure could allow design of candidate inhibitors.

The absence of salt-induced 1244G7 twitch inhibition when using treated assay dishes, as well as the reduced solubility of this strain as compared with the WT, indicates that there is an interaction between the non-glycosylated pilus surface and the hydrophobic polystyrene plate surface used in the standard assay. If this is the case, it is possible that the repetitive hydrophobic regions of the pilus lateral surface may interact directly with the elements in the pathogen’s environment. For example, the pilin glycan could prevent a non-specific interaction between pil of the pathogen and hydrophobic host matrix proteins such as fibronectin (Singh et al., 2010). In this environment an organism producing glycosylated pili would have, due to enhanced twitching motility and reduced non-specific lateral interactions, an advantage by enhancing pilus function. Alternatively, the absence of the glycan from a group II pilin could increase colonization at hydrophobic sites or on non-natural hydrophobic environment locations such as implant and catheter surfaces, leading to enhanced biofilm formation. Overall, these results indicate that a balance between the physical and chemical characteristics of the pilus lateral surface and the host colonization site is an important determinant in pathogen success. This is an important area of study that should be examined in future work.

In summary, this study has presented evidence that the pilin oligosaccharide has a role in maintaining function by moderating fibre interaction with its environment. These results suggest that even though the newly synthesized O-antigen repeating unit is primarily destined to become lipopolysaccharide and not the glycan moiety of the group I pilus, this glycan must also be considered a...
functionally integral component of the *P. aeruginosa* group I pilus since this structure expresses reduced functionality in its absence. Since the O-antigen repeating units are diverse in structure and physical properties, it can be questioned whether certain of these oligosaccharides are preferentially utilized in the production of group I pilin or if any of the structural types (Knirel, 1990; Knirel et al., 2006) are able to carry out the pilin glycan function. Further studies will be required to answer this question.

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