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PilO of *Pseudomonas aeruginosa* 1244: subcellular location and domain assignment

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Summary

PilO of *Pseudomonas aeruginosa* 1244 catalyses the attachment of an O-antigen repeating unit to the β -carbon of the pilin C-terminal residue, a serine. The present study was conducted to locate the regions of this enzyme important in catalysis and to establish the cellular location of the pilin glycosylation reaction. While PilO was not detectable in extracts of *P. aeruginosa* or *Escherichia coli*, even under conditions of overexpression, it was found that an intact MalE–PilO fusion protein was produced in significant amounts. This fusion complemented a *P. aeruginosa* 1244 mutant containing a *pilO* deletion and targeted to the cytoplasmic membrane of *E. coli*. Wzy and WaaL, enzymes that also utilize the O-antigen repeating unit as substrate, were found to share a sequence pattern with PilO even though these proteins have little overall sequence similarity. PilO constructs in which portions of this common sequence were deleted or altered by site-directed mutagenesis lacked pilin glycosylating activity. Deletions of segments downstream from the common region also prevented enzyme activity. Topology studies showed that the two PilO regions associated with enzyme activity were located in the periplasm. These results establish regions of this enzyme important for catalysis and present evidence that pilin glycosylation occurs in the periplasmic space of this organism.

Introduction

The pili of *Pseudomonas aeruginosa* are polar protein fibres that facilitate both adhesion and a type of surface-associated motility called twitching (Strom and Lory, 1993; Mattick, 2002), traits which contribute to the virulence of this opportunistic pathogen (Wood *et al.*, 1980; Comolli *et al.*, 1999). The pili of *P. aeruginosa* are categorized as type IV based on structural features of the monomeric subunit, pilin (Craig *et al.*, 2004), one of which is the N-methylation of the pilin N-terminal residue. Another post-translational modification of some of the type IV pilins is the covalent attachment of a glycan (Virji *et al.*, 1993; Castric, 1995; Voisin *et al.*, 2006). In the case of *P. aeruginosa* 1244 pilin, it has been shown that a single 666 Da trisaccharide which is identical with the O-antigen repeating unit of this strain's lipopolysaccharide is O-linked through the β -carbon of a serine found at the protein's C-terminus (Castric *et al.*, 2001; Comer *et al.*, 2002). Immunogold labelling studies using a glycan-specific monoclonal antibody have shown this saccharide to be surface-exposed over the entire fibre (Smedley *et al.*, 2005) where it strongly influences pilus hydrophilicity. Examination of a strain 1244 mutant that was unable to glycosylate pilin showed that the presence of the glycan is not required for piliation or for pilus retraction (Smedley *et al.*, 2005). However, competition studies, between strain 1244 and a glycosylation-deficient derivative of this organism, employing a mouse respiratory model, suggested that the pilin glycan has an important role in virulence (Smedley *et al.*, 2005).

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Studies using *P. aeruginosa* 1244 mutants defective in synthesis of the O-antigen indicated that the O-antigen biosynthetic pathway is the source of the pilin glycan (DiGiandomenico *et al.*, 2002). The absence of pilin glycosylation in a mutant that was unable to add the first sugar to the undecaprenol carrier lipid showed that the pilin glycan is attached by group transfer rather than by a stepwise reaction involving products of individual sugar pathways (DiGiandomenico *et al.*, 2002). These results suggested that the carrier lipid bound O-antigen repeating unit is the glycan substrate for the pilin glycosylation reaction. Pilin glycosylation resulting from expression of heterologous O-antigen gene clusters in strain 1244 confirmed these results (DiGiandomenico *et al.*, 2002), and, importantly, showed that the pilin glycosylation machinery has an extremely low glycan substrate specificity. Evidence has been presented that the general N-glycosylation system of *Campylobacter jejuni* displays a similar low glycan specificity (Feldman *et al.*, 2005). Recent experiments using truncated O-antigen precursors suggest that this substrate permissiveness in *P. aeruginosa* pilin glycosylation is likely due to the ability of the pilin glycosylation system to recognize common features of the reducing end component of the O-antigen repeating unit (Horzempa *et al.*, 2006a) while ignoring the remaining saccharide components. Studies on pilin substrate specificity in strain 1244 have shown that, unlike bacterial and eukaryotic N-glycosylation (Nita-Lazar *et al.*, 2005; Kowarik *et al.*, 2006), O-glycosylation of 1244 pilin does not recognize a specific sequence motif (Horzempa *et al.*, 2006b). Instead, the pilin glycosylation apparatus requires only a C-terminal serine (or threonine) and a charge-compatible pilus surface.

While prokaryotic protein glycosylation has been the subject of much study recently (Schmidt *et al.*, 2003; Szymanski and Wren, 2005), the transferases involved in forming the sugar-protein link have not received similar attention. Pilin glycosylation by *P. aeruginosa* 1244 requires a functional *pilO* gene which codes for the enzyme necessary for glycan attachment (Castric, 1995; Smedley *et al.*, 2005). Work with PilO has been hampered by the inability to produce detectable amounts of this protein and the resistance of this enzyme to standard methods of overexpression. We have shown in the current article that PilO can be expressed in a functional form, and in significant amounts, as a MalE fusion, thus allowing characterization of the pilin glycosylation enzyme to go forward. Work presented in this article indicated that PilO is located in the cytoplasmic membrane. In addition, mutagenesis experiments have located regions of this enzyme important in pilin glycosylation. Topological studies reveal that these PilO regions are located in the periplasmic space, providing evidence that pilin glycosylation occurs in this chamber.

Results

PilO structural organization

PilO has a predicted molecular weight of 51 003 kDa. The majority of the 461 residues making up this protein are hydrophobic (Fig. 1), suggesting a membrane location. Transmembrane helix analysis using TMHMM predicted that nine such regions make up 196 (74%) of the first 266 residues (Fig. 1). The remainder of PilO, residues 267 through 461, is, in general, less hydrophobic. In addition, approximately 66% of the latter region was predicted to extend into either the cytoplasm or the periplasm. Altogether, this arrangement suggested that the portion of PilO between residues 1 and 266 may serve primarily as a membrane anchor, while the rest of the protein could contain all or a large part of this enzyme's catalytic domain.

PilO expression

Initial attempts to physically detect PilO, a requirement for determination of subcellular location and topological studies, were unsuccessful. Although cloned *pilAO* operon under

control of a *tac* promoter produced strain 1244 pilin when expressed in *Escherichia coli*, no other novel protein was seen (results not shown). This is not surprising as *pilO* transcript production has been shown to be far below that of *pilA*, a response probably caused by a terminator-like structure seen between the *pilA* and *pilO* genes (Castric, 1995). To see if expression was reduced by regulatory regions 5' to *pilO* or within the initial coding portion of this gene, DNA coding for the first four residues (-MRIW-) of PilO was replaced with an in-frame segment coding for -MARIDP-. When this construct, pMBT100, was expressed in *E. coli* or *P. aeruginosa*, and analysed by polyacrylamide gel electrophoresis, no new protein was detected (results not shown). The hybrid gene was functional as expressing it in the *P. aeruginosa* 1244 mutant lacking the *pilO* gene resulted in pilin glycosylation (Smedley *et al.*, 2005). Attempts at expression as a histidine-tagged protein were uniformly unsuccessful (results not shown).

The finding that PilO resisted overexpression suggested that it might be an inherently unstable protein. In order to determine whether a protein fused to PilO could potentially increase expression through enhanced stability, a plasmid, pPAL100, was constructed that coded for a MalE–PilO hybrid. The gene construct was based on pMal-cR1 (Table 1), which contains DNA coding for MalE lacking a signal peptide. The fusion produced by pPAL100 would be expected to be composed of 390 residues of the N-proximal region of MalE which was joined at residue 5 of PilO and continued to the natural C-terminal residue (461) of the glycosylating protein. Expression of the empty vector produced a cytoplasmic protein of the expected size as MalE (Fig. 2A). Antigen was seen at the same relative position in a Western blot probed with a MalE-specific antiserum (Fig. 2B). A small amount of MalE antigen was found associated with the membrane fraction, possibly due to non-specific binding.

Induction of the *malE-pilO* of pPAL100 produced no cytoplasmic MalE and a membrane-associated protein of an apparent molecular weight of 79 kDa (Fig. 2A). A Western blot of a gel run under identical conditions (Fig. 2B) using anti-MalE serum as probe revealed a membrane-associated antigen of the same size as the novel membrane protein seen in Fig. 2A. Unfused MalE was not detectable (Fig. 2B). Altogether, these results indicate that PilO was detectable as a MalE fusion and that this construct was localized entirely in the cellular membrane fraction. As the MalE–PilO fusion is predicted to have a mass of 92 967 Da, it is clear that the protein fusion did not migrate as anticipated. This could be due to fusion degradation or to incomplete unfolding in the presence of SDS. To determine if degradation occurred in the PilO portion of the fusion, two pPAL100 deletion mutants were constructed. One, pMB322MAL, contained DNA coding for a transcription stop signal in frame after the codon for PilO residue 322 (see Fig. 1 for position). The other, pMB430MAL, had a transcriptional stop inserted in frame after the codon for PilO residue 430. When examined by Western blot (Fig. 2C), pMB322MAL produced a fusion with an apparent molecular weight of 69 kDa, while pMB430MAL produced what would appear to be a 76 kDa protein. These differences in apparent molecular weight correspond to the differences in fusion coding region size. These results suggested that the C-proximal region of the fusion produced by pPAL100 is likely intact as the deletions incorporated could not be expected to influence the size of a protein truncated to the degree suggested by the apparent size of the pPAL100 protein. It is possible that the fusion was degraded at the amino end, but it is more likely that unfolding during electrophoresis was incomplete due to protein hydrophobicity, leading to aberrant electrophoretic migration. It should be noted that while intact fusion was a major product, it is clear from the blots presented in Fig. 2 that protein degradation had also occurred.

The fusion protein produced from pPAL100 was purified by affinity chromatography and analysed by Western blot (results not shown). Here, a protein of identical apparent

molecular weight as that seen in Fig. 2B was seen. Attempted mass determination of the fusion using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) was unsuccessful possibly due to aggregation of this hydrophobic protein (results not shown). Digesting affinity-purified fusion with the endopeptidase factor Xa, which is specific for sequence found only at the MalE–PilO junction, reduced the apparent molecular weight of the immunoreactive component to that of the Maltose-Binding Protein standard (results not shown). Altogether, these results showed that the presence of MalE substantially stabilized PilO although fusion degradation products are apparent. In addition, these experiments provided evidence that PilO targeted the fusion to either the inner or outer membrane.

In order to determine if the fusion was enzymatically functional, DNA coding for this protein was transferred to a vector capable of replicating in *P. aeruginosa*. The ability of this plasmid, pMAQ, to produce the fusion in this organism was tested in the PilO-negative mutant of strain 1244 by Western blot using the anti-MalE antibody. Here, an approximately 79 kDa antigen was recognized (Fig. 3A), showing that the fusion was produced in *P. aeruginosa* in a manner similar to that seen in *E. coli*. The expression of the hybrid gene in this strain resulted in complementation of the PilO defect (Fig. 3B), which showed that the presence of MalE did not interfere with PilO catalytic activity and suggested that this enzyme targeted to its proper physiological location. As with expression in *E. coli*, the detected fusion appeared to be smaller than predicted, indicating possible incomplete protein unfolding during electrophoresis.

Subcellular location of PilO

Because the putative PilO membrane anchor region of the MalE–PilO fusion is intact, it could be expected that this protein construct would target normally. In order to determine precise subcellular location, *E. coli* expressing the fusion was fractionated as described in *Experimental procedures*. Individual fractions were analysed by Western blot using the MalE-specific polyclonal serum as probe. The fusion was found to be located almost entirely in the cytoplasmic membrane fraction (Fig. 4), a finding consistent with the predicted physical characteristics of PilO. Markers were used to determine whether the inner and outer membranes had been truly separated. Succinic dehydrogenase, an inner membrane marker, was found to have a specific activity of 329 units mg^{-1} in the inner membrane fraction and 45 units mg^{-1} for the outer membrane fraction. 3-Deoxy-D-manno-octulosonic acid (KDO), a component of lipopolysaccharide, should be found primarily in the outer membrane. Chemical analysis of the fractions for this molecule gave values of 50 and 305 ng of KDO per microgram of protein for the inner and outer membrane fractions respectively. These results indicate a high degree of separation of the two membrane fractions and suggest that the localization data of the fusion are valid. Overall, these results suggest that PilO functions at the cytoplasmic membrane where pilin glycosylation could potentially take place on either the inner or outer surfaces of this structure.

PilO catalytic domain

It has been previously noted (Power *et al.*, 2006) that PilO belongs to the Wzy_C family (PF04932) of proteins (Bateman *et al.*, 2004). Other members of this grouping are Wzy, the O-antigen polymerase, and WaaL, the enzyme that transfers the polymerized O-antigen to core-lipid A. Common features of these proteins (Fig. 5A) include size, hydrophobicity, number and relative position of numerous predicted transmembrane domains, as well as position of the Pfam identity domain (a region that exists in the putative catalytic portion of PilO). These findings are particularly interesting in that all three enzymes catalyse a transferase reaction that utilizes an undecaprenol-linked O-antigen saccharide as substrate. While functionally similar, a comparison of PilO primary structure with a number of Wzy and WaaL proteins reveals little overall sequence homology (results not shown). However, a

BLAST search using the Wzy_C domain of PilO (residues 275 through 325) as query produced many positive responses from open reading frames annotated as being PilO, Wzy or WaaL. In each case, the region corresponding to PilO residues 281 through 301 produced the strongest similarity (results not shown). As the sequences recognized in this search have not been functionally confirmed, this 21-residue region, as found in authentic proteins, was examined (Fig. 5B). Here, a strong similarity was seen in this region between PilO and four characterized proteins (two Wzy and two WaaL). While these proteins contain sequence homology to PilO, it must be noted that other functionally vetted Wzy_C proteins, the Wzy of strains PAO1 (Coyne and Goldberg, 1995; de Kievit *et al.*, 1995) or PA103 (Dean *et al.*, 1999), show no similarity in this region.

In order to determine if the conserved region was required for pilin glycosylation, two mutants (Fig. 6A) were prepared from cloned *pilO*. In the first (pMOR7), positions 281 through 287 (-RDVLWRD-) were deleted, while in the second (pR281A), residue 281 (R) was replaced with an alanine. When assayed by Western blot using a pilin-specific monoclonal antibody as probe it was found that neither of these mutants was able to complement the strain 1244 mutant in which the *pilO* gene was deleted (Fig. 6B). These results indicate that this region of similarity is required for enzyme function. As the Wzy_C enzymes share a common substrate, the lipopolysaccharide O-antigen repeating unit, this segment may be required for oligosaccharide transfer. However, it is also possible that it has an important structural, regulatory or targeting function.

We wished to determine whether residues in other portions of the putative catalytic region were important in pilin glycosylation. To do this, a construct was made that lacked DNA coding for PilO downstream from residues 322 (Fig. 6A). This plasmid, pMB322, was unable to support pilin glycosylation in the 1244 mutant lacking a functional *pilO* gene (Fig. 6B). In addition, constructs lacking DNA coding for PilO downstream of residues 430 or 453 (Fig. 6A) were likewise inactive (Fig. 6B).

It was important to show that the results described were not the result of interference with PilO expression or targeting. Because PilO from pMOR7 and pR281A were undetectable due to poor expression, the altered DNA of these constructs were incorporated into pPAL100 (producing pMOR7MAL and pR281AMAL) and tested by Western blot using a MalE-specific serum as probe. The mutated DNA from pMB453 was likewise moved into pPAL100 (producing pMB453MAL) and tested in the same way. In each case, membrane-targeted fusion of predicted size was produced in significant amounts (Fig. 7). Figure 2C has shown that the MalE–PilO fusion truncated at PilO positions 322 and 430 also produced membrane-targeted fusion. For comparison, a third mutant of the common region, pMOHis, in which six positions were replaced by histidine residues (Table 1), was shown to be unable to glycosylate pilin (results not shown). Poor expression with no completely intact fusion was seen when mutagenized DNA of this construct was moved to pPAL100 (producing pMOHisMAL) and tested (Fig. 7). These results indicate that the lack of activity of this construct was likely due to fusion instability caused by the presence of charged His residues in this region of the protein.

Altogether, these results support the hypothesis that the region containing residues 267 through 461 are necessary for glycosylation activity and may house at least a part of the PilO catalytic region. In addition, these experiments also provide evidence that, while the Wzy_C domain is necessary for pilin glycosylation, regions C-proximal to this structure, including residues near the C-terminus, are also required.

Topology of putative catalytic domain

In order to determine the location of the pilin glycosylation reaction, topological studies of the putative catalytic region were undertaken. Topological analysis of the Wzy from *Shigella flexneri* (Daniels *et al.*, 1998) and the WaaL of *Vibrio cholerae* (Schild *et al.*, 2005) has demonstrated that the Wzy_C region of these proteins extend into the periplasm. While several topology programs (e.g. $TMHMM$) indicated that the Wzy_C domain of PilO has a periplasmic location, the orientation of the regions downstream was predicted with less confidence. With this in mind, PhoA and LacZ fusions of the C-proximal regions of the MalE–PilO construct were prepared and analysed in order to determine actual topology. In the first series, PhoA was fused in frame at PilO positions 280, 290, 300, 310, 322, 353, 376, 403, 430, 440 and 453 (Fig. 8A). Western blots of membrane fractions using a monoclonal anti-PhoA antibody as probe showed that the majority of these fusions could be detected at anticipated apparent molecular weights (Fig. 8B), providing evidence that C-terminal fusions can, at least in some positions, further stabilize the MalE–PilO construct. When these constructs were tested in *E. coli* on X-Phos agar in the presence of inducer, fusions at PilO positions 280, 290, 300, 310, 322, 376, 430, 440 and 453 produced blue colonies while fusions 353 and 403 gave no colour reaction (results not shown). Quantification of these results by assay of whole cells for alkaline phosphatase activity is presented on Table 2. Here it can be seen that unambiguous activity was associated with PilO fusions 280, 290, 300, 310, 322, 376, 430, 440 and 453 indicating that these residues resided in the periplasm. The lack of PhoA activity by the fusion attached at PilO residues 353 and 403 suggested that these residues were located in the cytoplasm. That these two fusions were not detectable by Western blot (Fig. 8B) was consistent with this proposal as alkaline phosphatase could not fold correctly in the cytoplasm and would be subject to proteolysis. LacZ fusions were made at PilO positions 353 and 403 to further examine this point. The high level of LacZ activity seen with these fusions (Table 2) is consistent with a cytoplasmic location for fusions at both of these positions.

The $TMHMM$ analysis program predicted three transmembrane domains in the putative catalytic region (Figs 1 and 5A). In this model, the conserved residues 281 through 301, which have been shown to be necessary for glycosylation activity, would be in the periplasm. However, the C-proximal region (residues 453 through 461), which was also found to be necessary for activity, was predicted to be in the cytoplasm. In contrast, our results suggest that the putative catalytic region contains four transmembrane regions (Fig. 9), and that both regions necessary for catalysis reside in the periplasm. We propose that PilO residues 266 through approximately residue 327 form a periplasmic loop region, while positions 328 through 344 occupy a transmembrane segment. Residues 345 through 360 occupy a short cytoplasmic segment. The PilO region composed of residues 361 through 396 likely forms a transmembrane region which loops backwards exiting in the cytoplasm. This interpretation was supported by the PhoA fusion at position 376 which is in the centre of this approximately 36-residue region and which produced high alkaline phosphatase activity (Table 2). This segment was followed by another short cytoplasmic region composed of PilO residues 397 through 409. Another transmembrane region (positions 410 through 429) connected to another periplasmic region (PilO residues 430 through 461, the C-terminus). This interpretation is consistent with the ‘inside positive’ rule (von Heijne, 1992) in which charged residues of bacterial cytoplasmic membrane proteins occur primarily on the cytoplasm side where they are stabilized by the proton gradient. Altogether, these results indicate that approximately three-quarters of the non-membrane residues of this region exist in the periplasm, including the two regions required for pilin glycosylation. Thus, it is reasonable to suggest that pilin glycosylation takes place in this chamber and that the glycan substrate must be transported from the cytoplasm into this space in order for this reaction to occur.

Discussion

The present study has shown that PilO of *P. aeruginosa* 1244 is extremely difficult to detect, a phenomenon that is shared by related membrane proteins (Daniels *et al.*, 1998; Abeyrathne and Lam, 2007). N-terminal fusion of this protein with MalE, a relatively large protein, allows robust expression of catalytically active enzyme. Similarly, we have showed that several PilO mutants are expressed and targeted correctly as MalE fusions. In this arrangement, the stabilizing element is in the cytoplasm while we have shown that the putative PilO catalytic region is in the periplasm, an arrangement that may prevent interference with enzyme activity. This general approach could well have application in the study of other Wzy_C proteins, as well as other membrane proteins.

Experiments described in this article indicate that PilO is located in the cytoplasmic membrane and that both the Wzy_C domain and the C-proximal hydrophilic portion of this enzyme extend into the periplasm. As we have presented evidence that these PilO regions contain elements necessary for catalytic activity, it is reasonable to propose that pilin glycosylation occurs in this chamber. As previous evidence (Castric *et al.*, 2001; DiGiandomenico *et al.*, 2002) has been presented that undecaprenol-linked O-antigen repeating unit is the glycan substrate, the current results suggest that this glycolipid is transported to the periplasm as part of the O-antigen biosynthetic pathway where it serves also as substrate for pilin glycosylation. In this situation, PilO would operate in the same cellular environment as its two functional analogues, Wzy (the O-antigen repeating unit polymerase) and WaaL (the enzyme that transfers polymerized O-antigen to lipopolysaccharide core), where these three enzymes would likely compete for a common substrate, lipid-linked monomeric O-antigen. PilO, Wzy and WaaL all belong to the Pfam Wzy_C family meaning that they contain multiple transmembrane regions, are approximately the same size and share the characteristic Wzy_C domain in the same relative part of the protein. In addition, they are all transferases that attach a lipid-linked O-antigen repeating unit to a second substrate. In the case of PilO, the O-antigen is monomeric, while WaaL and Wzy utilize either monomeric or polymerized forms of the subunit. The common structural and functional features imply a phylogenetic relationship among these proteins, while general sequence dissimilarity indicates otherwise. Future work may reveal, as suggested in the present study, that sequence features are conserved in portions of the protein involved in catalysis.

While many similarities exist, PilO, WaaL and Wzy also differ in a number of respects. Wzy requires a specific intact O-antigen repeating unit for activity (Feldman *et al.*, 1999; Dean *et al.*, 2002; Horzempa *et al.*, 2006a), which is likely due to the need of this enzyme to recognize the terminal repeating unit sugar. On the other hand, both PilO (Horzempa *et al.*, 2006a) and WaaL (Feldman *et al.*, 1999) activities are non-specific with regards to the O-antigen structure and require only the first sugar of the repeating unit, a deoxyhexose containing an N-acetyl group at the number one carbon. In addition, these transferases each utilize a different co-substrate. PilO uses the terminal pilin residue, while WaaL employs the lipopolysaccharide core, and Wzy utilizes the terminal sugar of lipid-linked monomeric or polymeric O-antigen. These observations suggest that there are significant active site structural differences among these enzymes. It will be interesting to compare similarities of the glycan-binding regions of these enzymes, as well as the regions acting on co-substrate. In the case of PilO, as the C-proximal region of this enzyme is required for activity, it is possible that this segment is involved in pilin binding. Alternately, this part of PilO may be important in regulation or targeting, and so indirectly influence enzyme activity. Future work will be required to resolve these questions.

As mentioned above, the glycan specificity for the pilin glycosylation reaction lies in the first sugar of the O-antigen repeating unit (Horzempa *et al.*, 2006a), a saccharide that shows strong structural similarities among the O-antigens of *P. aeruginosa* (Hatano and Pier, 1998). This situation suggests that it might be possible to synthesize a low-molecular-weight molecule that would inhibit the glycosylation reaction, thereby preventing attachment of the O-antigen. This inhibition could likely be carried out *in vivo* because, as described in this article, the glycosylation reaction occurs in the periplasm into which a low-molecular-weight hydrophilic inhibitor would be able to easily enter by simple diffusion. As previous results from our lab (Smedley *et al.*, 2005) have shown that the presence of pilin O-antigen enhances virulence in the mouse respiratory model, glycosylation inhibition would be expected to have an antimicrobial effect. While not all *P. aeruginosa* strains produce glycosylated pilin, those that do are significantly represented among clinical isolates, and particularly among those found in respiratory infections (Kus *et al.*, 2004; Smedley *et al.*, 2005). As previously noted (Horzempa *et al.*, 2006a), the number one sugar of the O-antigen repeating unit from many pathogens share structural features with those of *P. aeruginosa*, suggesting that this strategy might be applied to other pathogens that produce glycosylated pilin. This approach, due to the above stated catalytic similarities among PilO, Wzy and WaaL, could also be applied to the inhibition of O-antigen subunit polymerization and O-antigen transfer to core-lipid A.

As there are a limited number of pilin subunits per *P. aeruginosa* cell, it is clear that only modest enzymatic activity is required for pilus glycosylation. For example, the pili of a typical cell containing five such fibres would be composed of approximately 3750 pilin subunits (Strom and Lory, 1993). We have determined that there is a ratio of about 2:1 between pilin contained in pilus fibres and the cell-bound form of *P. aeruginosa* 1244 (D. Furst and P. Castric, unpubl. obs.), suggesting a total of roughly 5600 subunits per cell. Thus, for a cell replicating every 45 min, only about one glycosylation event per second would be required, an extremely low catalytic demand. With this in mind, it is not surprising to see that the *pilO* gene produces far less transcript as compared with *pilA* (Castric, 1995). A further potential mechanism for the limitation of expression is the presence of 19 rare codons (Grocock and Sharp, 2002) within the first 30 PilO positions (P. Castric, unpubl. obs.). While these mechanisms likely contribute to low levels of enzyme synthesis, the extremely low amount of PilO present is also due to the instability of this protein. Results presented in this article have shown that PilO is only expressed in detectable amounts when it is fused to a relatively large protein (MalE). It is possible that PilO is susceptible to proteolysis in the absence of a stabilizing element that is present in limiting amounts. Examples of such stabilization can be found in *E. coli* where a number of proteins involved in formation of the bundle-forming pili are destabilized in the absence of specific piliation proteins (Ramer *et al.*, 2002; Crowther *et al.*, 2004). A possible physiological value for limiting PilO levels is the non-specific nature of this enzyme (DiGiandomenico *et al.*, 2002; Horzempa *et al.*, 2006a,b). With regards to protein substrate, only C-terminal serine or threonine and a compatible pilin surface is required (Horzempa *et al.*, 2006b). Production of PilO in amounts higher than presently seen could result in attachment of O-antigen repeating unit to the several predicted periplasmic proteins of *P. aeruginosa* that contain C-terminal Ser/Thr residues. Here, glycosylation could potentially interfere with protein function. It will be interesting to see if other prokaryotic protein glycosylating enzymes are also present in low amounts.

Experimental procedures

Culture conditions

All strains were routinely grown aerobically on LB plates or in broth cultures at 37°C (see Table 1 for strains employed). Broth cultures were grown on a rotatory shaker at 250 r.p.m.

The medium used for production of PhoA fusions was LB broth, while the medium for MalE and LacZ fusions was LB broth containing 0.2% glucose. The following antibiotics were employed at the indicated concentrations: ampicillin (Ap) at 100 $\mu\text{g ml}^{-1}$ for *E. coli*; carbenicillin (Cb) at 250 $\mu\text{g ml}^{-1}$ for *P. aeruginosa* 1244; tetracycline (Tc) at 15 $\mu\text{g ml}^{-1}$ for *E. coli* and 50 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*. Isopropyl β -D-thiogalactopyranoside (IPTG) was employed at a concentration of either 0.1 mM or 0.3 mM for *E. coli* and 5 mM for *P. aeruginosa*. 5-Bromo-4-chloro-3-indolyl Phosphate (X-Phos) was used at a concentration of 60 $\mu\text{g ml}^{-1}$. O-Nitrophenyl- β -D-galactopyranoside (ONPG) was 750 $\mu\text{g ml}^{-1}$.

Plasmid construction

Plasmids employed or constructed are listed in Table 1. In order to construct pPAL100, pPAC124 was digested with NheI and the DNA ends produced were polished using Klenow fragment. This DNA was ligated with a BamHI phosphorylated linker (Table 3) which reformed the NheI site. The product of this reaction was then digested with BamHI and HindIII, and the resulting fragment containing *pilO* DNA ligated with pMal-cRI digested with the same enzymes. pMAQ was constructed by digesting pPAL100 with HpaI and HindIII. The fragment containing the DNA fusion of *malE* and *pilO* was ligated with pMMB66EH digested with SmaI and HindIII. All junctions of constructs produced in this study were confirmed by nucleotide sequencing.

pMOHis was constructed by first replacing the DNA of pPAC124 coding for PilO residues 281 through 286 (-RDVLWR-) with DNA coding for six histidines using ExSite mutagenesis (Stratagene). Primers used (EmP280F, EmP280R) are given in Table 3. Mutagenesis was confirmed by nucleotide sequencing. The EcoRI/HindIII digestion fragment containing the *pilAO* operon was ligated with pMMB66EH cut with these same enzymes. During the sequence confirmation procedure a clone was observed that contained an in-frame deletion lacking DNA coding for PilO residues -RDVLWRD-. The *pilAO* segment of this construct was moved to pMMB66EH, thus producing pMOR7. MalE fusions of the mutagenized DNA were prepared by digesting either pMOHis or pMOR7 with NheI and HindIII and ligating the fragment produced with pPAL100 digested with the same enzymes.

pMB322 was constructed by ligating the BamHI/PstI fragment of pPAC202 containing *pilA* and truncated *pilO* with pRK404 digested with the same enzymes. PilO sequence (Fig. 1) terminated at residue 322 (Q). Construction added four residues (PSLA) before a stop codon was reached. pMB322MAL was constructed by amplifying a portion of the *pilO* gene using primers MEPOF2 (which hybridizes upstream from the *malE-pilO* junction region) and 322SCR. The latter primer binds at the PstI region and contains a stop after the codon for residue 322 and a subsequent HindIII site. The resulting DNA was ligated with pCR2.1-TOPO, digested with BamHI and HindIII and ligated with pPAL100 digested with these same enzymes.

To construct pMB430, a 0.638 kb segment of pET28a DNA containing the XhoI site and the His-tag region was amplified using primers PETF and PETR (which contained a HindIII site). The resulting DNA was ligated with pCR2.1-TOPO. The 0.55 kb fragment produced by digestion of this plasmid with XhoI and HindIII was ligated with pMAQ digested with the same enzymes. The PilO coding region of this construct terminated at residue 430 and was followed by DNA coding for six histidines and a stop codon. Construction of pMB430MAL was carried out using the same general strategy for making pMB322MAL. The sole difference was that the downstream primer (430SCR) hybridized in the region coding for PilO residue 430. In order to construct pMB453, a section of pMBT100 DNA was amplified with primer PIO453F, which hybridized upstream of *pilO*, and primer PIO453R (Table 3), which contained a HindIII site and which hybridized within the terminal

coding region of *pilO*. This fragment produced was ligated with pCR2.1-TOPO. The intermediate construct produced was digested with PstI and HindIII and ligated with pMAQ cut with the same enzymes. This construct resulted in termination of the PilO coding region at residue 453 and was followed by a stop codon. Construction of pMB453MAL was carried out using the same general strategy for making pMB322MAL and pMB430MAL. The sole difference was that the downstream primer (453SCR) hybridized in the region coding for PilO residue 453.

Construction of the MalE–PilO–PhoA fusions began with amplification of pPAL100 using primer MEPOF (Table 3), which hybridizes upstream of the MalE coding region, and primer PIO280R, which hybridized in the region containing the codon for residue 280 of PilO. The upstream primer had an incorporated XbaI site, while the downstream primer contained HindIII. The DNA produced was ligated with pCR2.1-TOPO. This plasmid, when digested with XbaI and HindIII and then ligated with pRMCD28 (digested with the same enzymes), produced pMAP280, a construct in which MalE–PilO was fused in frame with PhoA at PilO residue 280. pMAP290 was produced by first amplifying a region of pPAL100 using primer MEPOF2 which hybridized just upstream of the *malE-pilO* junction, and primer PIO290R, which contained a HindIII site, and which hybridized in the region of *pilO* containing the codon for residue 290. This DNA was ligated with pCR2.1-TOPO, and digested with HindIII and BamHI, which recognizes a unique site within the *malE-pilO* junction. The resulting fragment was ligated with pMAP280 cut with these same enzymes. The constructs, pMAP300, pMAP310, pMAP322, pMAP353, pMAP376, pMAP403, pMAP430 and pMAP440, were produced using the same general strategy as that used for pMAP290. Here downstream primers PIO300R, PIO310R, PIO322R, PIO353R, PIO376R, PIO403R, PIO430R and PIO440R were employed. pMAP453 was produced by first digesting the TOPO intermediate used in construction of pMB453 with NheI and HindIII. The fragment containing *pilO* DNA was ligated with pMAP280 cut with these same enzymes. The resulting construct produced MalE–PilO fused in frame with PhoA at PilO residue 453. All *pilO-phoA* junctions were confirmed by nucleotide sequencing.

Construction of the MalE–PilO–LacZ fusions began with amplification of pPAL100 using primers MEPOF, which hybridized upstream of the MalE coding region and which contained an XbaI site, and POZ353R, which hybridized in the region coding for residue 353 of PilO and which had an XmaI site. The DNA produced was ligated with pCR2.1-TOPO. The plasmid produced was digested with XbaI and XmaI and ligated with pRMCD70 cut with these same enzymes. This construct, pMAZ353, produced MalE–PilO fused in frame with LacZ at PilO residue 353. pMAZ403 was produced using the same general strategy as that used for pMAZ353. Here a downstream primer, POZ403R (which contained an XmaI site), was used in place of POZ353R. All *pilO-lacZ* junctions were confirmed by nucleotide sequencing.

Site-directed mutagenesis

This procedure was performed using the Quick Change II Site-directed Mutagenesis Kit (Stratagene) as directed by the manufacturer. The complementary primers used were 281RAF and 281RAR (Table 3). Alanine replacement of arginine 281 codon of the *pilO* of pPAC124 was carried out. DNA containing the *pilAO* operon was removed by digestion with EcoRI and HindIII and ligated with pMMB66EH digested with the same enzymes producing pR281A. Construction of pR281AMAL used the same strategy as that employed for the construction of pMOHisMAL and pMOR7MAL.

Purification of MalE–PilO by affinity chromatography

The protocol for extraction and purification of the MalE–PilO fusion was that described by New England Biolabs. Briefly, IPTG was added to a 1 l log-phase culture of *E. coli* TB1 (pPAL100) to a concentration of 0.3 mM which was then grown to an A_{600} of between 0.300 and 0.600. The cells were then harvested by centrifugation, suspended in lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM β -mercaptoethanol, 10 mM EDTA and 10 mM EGTA) and disrupted by sonication. This material was clarified by centrifugation and the supernatant fluid passed through a 2.5×10 cm amylose column (New England Biolabs). After washing with 180 ml of column buffer (New England Biolabs), the bound protein was eluted with fifteen 3 ml fractions of column buffer containing 10 mM maltose.

Subcellular fractionation of *E. coli*

A 1 l quantity of LB broth containing ampicillin, inoculated with 10 ml of an *E. coli* TB1 (pPAL100) overnight culture, was grown to an A_{600} value of 0.5. IPTG was added to 0.3 mM and the culture incubated for 2 additional hours, at which time cells were harvested by centrifugation at 4080 *g* at 4°C for 15 min. From this point on fractionation followed the protocol of Osborn and Munson (1974). Briefly, cells were suspended in ice-cold 10 mM Tris-HCl (pH 7.8) containing 0.75 M sucrose at final density of 7×10^9 cells ml⁻¹. Lysozyme was added to final concentration of 100 μ g ml⁻¹ and incubated on ice for 2 min. Conversion to spheroplast form was performed by slowly (drop wise) diluting the cells with 2 volumes of ice-cold 1.5 mM EDTA (pH 7.5) added slowly with stirring over a period of 10 min at 4°C. Conversion to spheroplasts was confirmed by light microscopy, at which point these cells were removed by centrifugation 5000 r.p.m. (4080 *g* at 4°C for 15 min). The resulting supernatant contained the periplasmic proteins and the outer membrane fraction. Outer membranes were pelleted by centrifugation at 27 500 r.p.m. (100 000 *g*) for 3 h using an SW-28 swinging bucket rotor (Beckman Instruments). The resulting supernatant fluid contained the periplasmic proteins. Spheroplasts were suspended in 25 ml of lysis buffer and briefly sonicated. Resulting cellular debris was removed by centrifugation at 5000 *g* for 15 min at 4°C. Inner membranes were then separated from the soluble fraction (cytoplasmic) by centrifugation at 100 000 *g* for 1.5 h using an SW-60 Ti swinging bucket rotor (Beckman Instruments). Lysis buffer was used to re-suspend all the fractions. Protein concentration was determined by Bio-Rad protein assay. Succinic dehydrogenase activity and 3-deoxy-D-manno-octulosonic acid levels were determined using previously published methods (Osborn *et al.*, 1972).

Fusion assays

Growth and harvesting of cells containing the PhoA and LacZ fusions, as well as enzymatic assay of the alkaline phosphatase and β -galactosidase, were performed as described by Daniels *et al.* (1998). Incubation was for 30 min at 37°C. Absorbance values were measured using a 96-well Bio-Rad Model 3550 Microplate Reader. Values presented are the mean of triplicate determinations, corrected for background using cells harbouring pRMCD28 and pRMCD70 for PhoA and LacZ fusions respectively. PhoA and LacZ enzyme units were calculated using a standard equation (Manoil, 1991).

Electrophoresis and Western blot methods

SDS-polyacrylamide electrophoresis and electroblotting of pilin to nitrocellulose paper were carried out as described previously (DiGiandomenico *et al.*, 2002). Anti-pilin monoclonal 5.44 (Castric *et al.*, 2001) was used as probe. FITC-labelled goat anti-mouse IgG (Sigma-Aldrich) was used as secondary antibody, which was detected by fluorescence using a

Molecular Dynamics 595 fluorimeter employing an excitation wavelength of 488 nm and a 530 DF30 absorbance filter.

To prepare cells for electrophoretic analysis of the MalE–PilO fusion, 100 ml of medium containing Ap was inoculated with 1 ml of an overnight culture in the same medium. This culture was grown to an A_{600} of 0.5, at which point IPTG was added to a concentration of 0.1 mM. These cells were incubated with agitation for 1 h at 25°C, and then were harvested by centrifugation. The pellet was washed twice with ice-cold phosphate-buffered saline and re-suspended in lysis buffer. Lysozyme was added to final concentration of 100 $\mu\text{g ml}^{-1}$ and incubated on ice for 30 min. This cell suspension was sonicated on ice with three 15 s bursts using a Biosonik III sonicator (intensity setting of 55, Bronwill Scientific). Cell debris and unbroken cells were removed by centrifugation at 6000 g for 15 min at 4°C in a Sorvall SS-34 rotor. The supernatant fluid was centrifuged at 30 000 r.p.m. for 1.5 h using an SW-60 Ti swinging bucket rotor (Beckman Instruments). The pellet, which contained the membrane fraction, was re-suspended in ice-cold PBS and ultracentrifuged as above. This step was repeated and the final pellet was re-suspended in 200 μl of lysis buffer. Protein levels were determined using the BCA assay (Pierce). Fractions were diluted in loading buffer, heated for 10 min at 37°C, and loaded, in an amount of 30 μg on either a 7.5% or a 10% T Tris/Glycine gel. Electrophoresis was carried out at 125 V at 4°C. The separated proteins were stained with Coomassie Brilliant Blue or electroblotted onto a nitrocellulose membrane for 25 min at 100 V, after which they were blocked for 25 min at room temperature, and were probed overnight at room temperature using the anti-Maltose-Binding Protein polyclonal antibody (New England Biolabs). An FITC-labelled secondary antibody was used as described above. Electrophoresis and Western blotting of affinity purified MalE–PilO fusion used the same protocols as described here. Analysis of the MalE–PilO fusion when produced in *P. aeruginosa* 1244G7 was identical as that described here except that Cb was the selective agent and 5 mM IPTG was used for induction.

The PhoA fusion samples were prepared by inoculating 100 ml of medium containing Ap with an overnight culture of *E. coli*, containing the constructs of interest, growing on the same medium. This culture was grown for 1 h at which point IPTG was added to 0.3 mM and the culture grown for 5 h. A membrane fraction was prepared for each sample as described above. Electrophoresis, electroblotting and detection of reactive proteins employed the same protocols described for the MalE–PilO fusion except that a 7.5% T Tris/Glycine gel was used. Each lane was loaded with 20 μg of protein. The primary antibody used (Sigma-Aldrich) was a monoclonal preparation directed against *E. coli* PhoA.

Sequence analysis

Sequence analysis programs are found at the following websites: Pfam (Bateman *et al.*, 2004), <http://pfam.janelia.org/>; TMHMM, <http://www.cbs.dtu.dk/services/TMHMM-2.0>.

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References

- Abeyrathne PD, Lam JS. WaaL of *Pseudomonas aeruginosa* utilizes ATP in *in vitro* ligation of O antigen onto lipid A-core. *Mol Microbiol.* 2007; 65:1345–1359. [PubMed: 17697256]
- Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, et al. The Pfam protein families database. *Nucleic Acids Res.* 2004; 32:D138–D141. [PubMed: 14681378]

- Bolivar F, Backman K. Plasmids of *Escherichia coli* as cloning vectors. *Meth Enzymol.* 1979; 68:245–267. [PubMed: 232214]
- Castric P. *pilO*, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology.* 1995; 141:1247–1254. [PubMed: 7773418]
- Castric P, Cassels FJ, Carlson RW. Structural characterization of the *Pseudomonas aeruginosa* 1244 pilin glycan. *J Biol Chem.* 2001; 276:26479–26485. [PubMed: 11342554]
- Castric PA, Sidberry HF, Sadoff JC. Cloning and sequencing of the *Pseudomonas aeruginosa* 1244 pilin structural gene. *Mol Gen Genet.* 1989; 216:75–80. [PubMed: 2499765]
- Comer JE, Marshall MA, Blanch VJ, Deal CD, Castric P. Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect Immun.* 2002; 70:2837–2845. [PubMed: 12010970]
- Comolli JC, Hauser AR, Waite L, Whitchurch CB, Mattick JS, Engel JN. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity *in vitro* and virulence in a mouse model of acute pneumonia. *Infect Immun.* 1999; 67:3625–3630. [PubMed: 10377148]
- Coyne MJ, Goldberg JB. Cloning and characterization of the gene (*rfc*) encoding O-antigen polymerase of *Pseudomonas aeruginosa* PAO1. *Gene.* 1995; 167:81–86. [PubMed: 8566816]
- Craig L, Pique ME, Tainer J. Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol.* 2004; 2:363–378. [PubMed: 15100690]
- Crowther LJ, Anantha RP, Donnenberg MS. The inner membrane subassembly of the enteropathogenic *Escherichia coli* bundle-forming pilus machine. *Mol Microbiol.* 2004; 52:67–79. [PubMed: 15049811]
- Daniels C, Vindurampulle C, Morona R. Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rcf/Wzy). *Mol Microbiol.* 1998; 28:1211–1222. [PubMed: 9680210]
- Dean CR, Franklund CV, Retief JD, Coyne MJ, Hatano K, Evans D, et al. Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. *J Bacteriol.* 1999; 181:4275–4284. [PubMed: 10400585]
- Dean CR, Datta A, Carlson RW, Goldberg JB. WbjA adds glucose to complete the O-antigen trisaccharide repeating unit of the lipopolysaccharide of *Pseudomonas aeruginosa* serogroup O11. *J Bacteriol.* 2002; 184:323–326. [PubMed: 11741875]
- DiGiandomenico A, Matewish MJ, Bisailon A, Stehle JR, Lam JS, Castric P. Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: specificity of glycan substrate. *Mol Microbiol.* 2002; 46:519–530. [PubMed: 12406226]
- Ditta G, Schmidhauser TJ, Yakobson E, Lu P, Liang XW, Finlay DR, et al. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid.* 1985; 13:149–153. [PubMed: 2987994]
- Feldman MF, Marolda CL, Monteiro MA, Perry MB, Parodi AJ, Valvano MA. The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O-antigen assembly is independent of the chemical structure of the O-repeat. *J Biol Chem.* 1999; 274:35129–35138. [PubMed: 10574995]
- Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, et al. Engineering N-linked protein glycosylation with diverse O-antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci USA.* 2005; 102:3016–3021. [PubMed: 15703289]
- Furste JP, Pansegrau W, Blocker FR, Scholz P, Bag-dasarian M, Lanka E. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene.* 1986; 48:119–131. [PubMed: 3549457]
- Grocock RJ, Sharp PM. Synonymous codon usage in *Pseudomonas aeruginosa* PAO1. *Gene.* 2002; 289:131–139. [PubMed: 12036591]
- Hatano K, Pier GB. Complex serology and Immune response of mice to variant high-molecular-weight O polysaccharides isolated from *Pseudomonas aeruginosa* serogroup O2 strains. *Infect Immun.* 1998; 66:3719–3726. [PubMed: 9673254]
- von Heijne G. Membrane protein structure prediction. Hydrophobicity analysis and positive-inside rule. *J Mol Biol.* 1992; 225:487–494. [PubMed: 1593632]
- Horzempa J, Dean CR, Goldberg JB, Castric P. *Pseudomonas aeruginosa* 1244 pilin glycosylation: glycan substrate recognition. *J Bacteriol.* 2006a; 188:4244–4252. [PubMed: 16740931]

- Horzempa J, Comer JE, Davis SA, Castric P. Glycosylation substrate specificity of *Pseudomonas aeruginosa* 1244 pilin. J Biol Chem. 2006b; 281:1128–1136. [PubMed: 16286455]
- de Kievit TR, Dasgupta T, Schweizer H, Lam JS. Molecular cloning and characterization of the *rflC* gene of *Pseudomonas aeruginosa* (serotype O5). Mol Microbiol. 1995; 16:565–574.
- Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, et al. Definition of the bacterial N-glycosylation site consensus sequence. EMBO J. 2006; 25:1957–1966. [PubMed: 16619027]
- Kus JV, Tullis E, Cvitkovitch DG, Burrows LL. Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. Microbiology. 2004; 150:1315–1326. [PubMed: 15133094]
- Manoil C. Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. Methods Cell Biol. 1991; 35:61–75. [PubMed: 1943817]
- Mattick JS. Type IV pili and twitching motility. Annu Rev Microbiol. 2002; 56:289–314. [PubMed: 12142488]
- Nita-Lazar M, Wacker M, Schegg B, Amber S, Aebi M. The N-X-S/T consensus sequence is required but not sufficient for bacterial N-linked protein glycosylation. Glycobiology. 2005; 15:361–367. [PubMed: 15574802]
- Osborn MJ, Munson R. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Meth Enzymol. 1974; 31:642–653. [PubMed: 4608978]
- Osborn MJ, Gander JE, Parisi E, Carson J. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J Biol Chem. 1972; 247:3962–3972. [PubMed: 4555955]
- Power PM, Seib KL, Jennings MP. Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. Biochem Biophys Res Commun. 2006; 347:904–908. [PubMed: 16870136]
- Ramer SW, Schoolnik GK, Wu CY, Hwang J, Schmidt SA, Bieber D. The type IV pilus assembly complex: biogenic interactions among the bundle-forming pilus proteins of enteropathogenic *Escherichia coli*. J Bacteriol. 2002; 184:3457–3465. [PubMed: 12057939]
- Schild S, Lamprecht AK, Reidl J. Molecular and functional characterization of O-antigen transfer in *Vibrio cholerae*. J Biol Chem. 2005; 280:25936–25947. [PubMed: 15908430]
- Schmidt MA, Riley LW, Benz I. Sweet new world: glycoproteins in bacterial pathogens. Trends Microbiol. 2003; 11:554–561. [PubMed: 14659687]
- Smedley JGI, Jewell E, Roguskie J, Horzempa J, Seyboldt A, Beer-Stolz D, Castric P. Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. Infect Immun. 2005; 73:7922–7931. [PubMed: 16299283]
- Strom MS, Lory S. Structure–function and biogenesis of the type IV pili. Annu Rev Microbiol. 1993; 47:565–596. [PubMed: 7903032]
- Szymanski CM, Wren BW. Protein glycosylation in bacterial mucosal pathogens. Nat Rev Microbiol. 2005; 3:225–237. [PubMed: 15738950]
- Virji M, Saunders JR, Sims G, Makepeace K, Maskell D, Ferguson DJP. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol Microbiol. 1993; 10:1013–1028. [PubMed: 7934852]
- Voisin S, Kus JV, Houliston S, St-Michael F, Watson D, Cvitkovitch DG, et al. Glycosylation of *Pseudomonas aeruginosa* strain Pa5196 type IV pilins with mycobacterial-like {alpha}-1,5 linked D-Araf oligosaccharides. J Bacteriol. 2006; 189:151–159. [PubMed: 17085575]
- Wood DE, Strauss DC, Johanson WG, Berry VK, Bass JA. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect Immun. 1980; 29:1146–1151. [PubMed: 6107276]

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MRIWLAWERM GRASRTILLL LISILLSPV VYCGVSKNWH DQQRILQLVV 50
LSGSSLLLLF SSRLSFARM VQVTLLVILG LGSVSAFLSA NPSWAFKEWS 100
VFAGLMLFSF NISASPEWVR RIALWGVVVL GGFECYQFLI SYLAAFVSGI 150
RELNPRVLLS GFSNVRTMGQ FQAMLLPIMA ALGLYLRETG RFRLSWLVML 200
LLAIQWCISF ALAGRGLWLG FAVAHLCW IGPVGRRLI VQLSAAFVGL 250
ALYFLLMVAL PTWLGIDMTL MSGMRSGLSL RDVLWRDAG MFVAHPLLGV 300
GPMHFSAVPN SVGAHPHQL LQWFAEWGGA AGLLVVGLMT LGLLRGARYL 350
REQGDPMDAG LWLALVSVLV LAQVDGVFVM PFTQTVLALL VGIAMARWSK 400
PVVPSPAQRW LCRGLAVVVI VVLGRVLLLE VPGLTAAEER YLEIHGGGEA 450
PRFWIQGWIP M 461

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Fig. 1.

Primary structure of *P. aeruginosa* 1244 PilO. The sequence in bold and underlined represents predicted transmembrane regions (TMHMM).

Fig. 2.

Expression of MalE–PilO fusion in *E. coli*.

A. SDS-PAGE gel stained with Coomassie brilliant blue in which membrane or cytoplasm fractions of cell grown in the presence or absence of 0.1 mM IPTG were assayed. An equal amount of protein (30 μ g) was added to each lane. pPAL100 codes for the MalE–PilO fusion. pMal-cR1 is the empty vector.

B. Western blot of a gel produced under conditions identical to the one shown in (A). Antibody probe was the anti-MalE polyclonal serum.

C. Western blot of MalE–PilO fusions containing deletions at the PilO C-terminus. The antibody probe was the anti-MalE polyclonal serum. An equal amount of protein (0.2 μ g) was added to each lane.

Fig. 3.

Expression of the Male-PilO fusion in *P. aeruginosa* 1244G7.

A. Western blot of membrane fractions of strain 1244G7 carrying the plasmid (pMAQ) coding for the protein fusion or the empty vector (pMMB66EH). Antibody probe was the anti-Male polyclonal serum.

B. Western blot of extracts of strain 1244G7 carrying pMAQ, pMMB66EH or pMBT100. These cultures were grown under identical conditions. Anti-pilin monoclonal 5.44 was used as probe. M_r, molecular mass.

Fig. 4. Subcellular localization of the MalE-PilO fusion as expressed in *Escherichia coli*. Cell fractions were prepared as described in *Experimental procedures* and were then subjected to SDS-PAGE, blotted to nitrocellulose paper and probed with the anti-MalE polyclonal serum. An equal amount of protein (30 μ g) was added to each lane.

Fig. 5.

Structural comparison of PilO, Wzy and WaaL.

A. Comparison of the predicted transmembrane and Wzy_C domains of the PilO of *P. aeruginosa* 1244 with the Wzy and WaaL of *E. coli*. The corner-filled rectangles are predicted transmembrane regions ($_{TMHMM}$). The centre-filled rectangles signify the Pfam Wzy_C domain (Bateman *et al.*, 2004) of these proteins.

B. Comparison of the conserved region of the PilO Wzy_C domain of *P. aeruginosa* 1244 with analogous regions of the WaaL of *P. aeruginosa* PAO1 and *Vibrio cholerae* and the Wzy of *E. coli* and *Pseudomonas putida*. Accession numbers for protein sequences in this figure are: *P. aeruginosa* 1244 (PilO), CAA58769; *P. aeruginosa* PAO1 (WaaL), AAG08384; *V. cholerae* (WaaL), AAL76926; *E. coli* (Wzy), AAD21566; *P. putida* (Wzy), AAF15969.

Fig. 6.

The effect of site-directed mutagenesis and sequence deletions of the *P. aeruginosa* 1244 *pilO* on pilin glycosylation.

A. Mutant location and identification. Arrows pointing downward towards the dashed lines indicate internal deletion of residues 281 through 287. Arrow above residue 281 indicates the position of site-directed mutagenesis. Delta symbols indicate the last PilO residue of the C-proximal deletion mutants. The construct designations are in brackets next to the mutation position.

B. Western blot analysis of the effect of mutagenesis of *pilO* gene on pilin glycosylation. Expression was tested in strain 1244G7, a mutant lacking a functional *pilO* gene. Blots were probed with anti-pilin monoclonal 5.44. Non-glycosylated pilin was produced by strain 1244G7 carrying an empty vector (pMMB66EH). Glycosylated pilin was produced by 1244G7 carrying a plasmid (pMBT100) expressing a functional *pilO* gene. Authentic 1244 pilin was used as a second positive control.

Fig. 7. Determination of expression and subcellular location of *P. aeruginosa* 1244 *pilO* site-directed and deletion mutations. Construction of these plasmids is described in *Experimental procedures*. Expression was carried out in *E. coli*. Western blots using anti-MalE serum was carried out on membrane fractions of these strains. Equal amounts of protein (20 μ g) were added to each lane.

Fig. 8.

Expression of the Male–PilO–PhoA fusions in *E. coli*.

A. PhoA fusion positions. Delta symbol and number indicate the position at which PhoA is fused. The Wzy_C conserved region sequence (in bold) is included for position reference.

B. Western blot analysis of membrane fractions from cells expressing the fusions indicated in (A). Equal amounts of protein (20 μ g) were added to each lane. The lane designations indicate vector control (pRMCD28) or fusion position. The asterisks indicate the presence of weak bands produced by fusions 300 and 430. A monoclonal anti-PhoA serum was used as probe.

Fig. 9. Topological model of PilO putative catalytic region. Black diamonds indicate the PhoA fusion positions. The black triangles are the sites of LacZ fusion.

Table 1

Bacteria and plasmids used in this study.

Organism	Description	Source or reference
Bacteria		
<i>P. aeruginosa</i>		
1244	Wild-type strain	Castric <i>et al.</i> (1989)
1244G7	$\Delta pilO$	Smedley <i>et al.</i> (2005)
<i>E. coli</i>		
HB101	<i>supE44 hsdS20 recA13 ara-14 proA2 lac Y1 galK2 rpsL2 xyl-5 mtl-1</i>	Bolivar and Backman (1979)
TB1	<i>araΔ(lac proAB) rpsL (Φ80 lacZΔM15) hsdR</i>	New England Biolabs
Plasmids		
pCR2.1-TOPO	Ap ^r , 3.9 cloning vector	InVitrogen
pET28a	Km ^r , His tag vector	Novagen
pMMB66EH	Ap ^r , broad host range vector	Furste <i>et al.</i> (1986)
pMal-cRI	Ap ^r , MalE fusion vector. Product directed to cytoplasm	New England Biolabs
pPAC124	Ap ^r , 2.3 kb insert containing strain 1244 <i>pilAO</i> under <i>tac</i> promoter in pUC18	Castric (1995)
pPAC46	Ap ^r , pMMB66EH containing strain 1244 <i>pilAO</i> under <i>tac</i> promoter	Castric (1995)
pMBT100	Ap ^r , 1.2 kb insert containing 1244 <i>pilO</i> under <i>tac</i> promoter in pMMB66EH	Smedley <i>et al.</i> (2005)
pPAC202	Tc ^r , subclone of a cosmid clone containing <i>pilA</i>	Castric <i>et al.</i> (1989)
pRK404	Tc ^r , low-copy-number expression vector	Ditta <i>et al.</i> (1985)
pPAL100	Ap ^r , MalE–PilO fusion	This study
pMAQ	Ap ^r , pMMB66EH with HpaI–HindIII segment containing <i>malE–pilO</i> from pPAL100	This study
pR281A	Ap ^r , pPAC46 in which R281 of PilO has been converted to A	This study
pMOR7	Ap ^r , pPAC46 in which <i>pilO</i> residues 281–287 have been deleted	This study
pMOHis	Ap ^r , pPAC46 in which <i>pilO</i> residues 281–286 have been replaced with a 6xHis tag	This study
pMB322	Tc ^r , pRK404 into which was inserted the 1244 <i>pilAO</i> operon lacking <i>pilO</i> residues 323–461	This study
pMB430	Ap ^r , pMAQ lacking the final 31 residues of PilO	This study
pMB453	Ap ^r , pMAQ lacking the final 8 residues of PilO	This study
pR281AMAL	Ap ^r , pPAL100 containing pR281A mutated DNA	This study
pMOR7MAL	Ap ^r , pPAL100 containing pMOR7 mutated DNA	This study
pMOHisMAL	Ap ^r , pPAL100 containing pMOHis mutated DNA	This study
pMB322MAL	Ap ^r , pPAL100 containing pMB322 deletion	This study
pMB430MAL	Ap ^r , pPAL100 containing pMB430 deletion	This study
pMB453MAL	Ap ^r , pPAL100 containing pMB453 deletion	This study
pRMCD28	Ap ^r , PhoA fusion vector	Daniels <i>et al.</i> (1998)
pMAP280	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 280	This study
pMAP290	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 290	This study
pMAP300	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 300	This study
pMAP310	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 310	This study
pMAP322	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 322	This study

Organism	Description	Source or reference
pMAP353	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 353	This study
pMAP376	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 376	This study
pMAP403	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 403	This study
pMAP430	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 430	This study
pMAP440	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 440	This study
pMAP453	Ap ^r , pRMCD28. PhoA fusion at MalE–PilO residue 453	This study
pRMCD70	Ap ^r , LacZ fusion vector	Daniels <i>et al.</i> (1998)
pMAZ353	Ap ^r , pRMCD70. LacZ fusion at MalE–PilO residue 353	This study
pMAZ403	Ap ^r , pRMCD70. LacZ fusion at MalE–PilO residue 403	This study

Table 2

PilO fusion reporter enzyme activity.

Construct	PilO fusion position residue number	PhoA activity units	LacZ activity units
None	–	0.0	0.0
pRMCD28	–	0.0	–
pRMCD70	–	–	0.0
pMAP280	280	1418.2	–
pMAP290	290	2977.6	–
pMAP300	300	297.3	–
pMAP310	310	610.0	–
pMAP322	322	962.1	–
pMAP353	353	0.0	–
pMAZ353	353	–	1426.8
pMAP376	376	3050.4	–
pMAP403	403	0.0	–
pMAZ403	403	–	3239.8
pMAP430	430	938.0	–
pMAP440	440	4596.0	–
pMAP453	453	1820.0	–

Table 3

Oligonucleotides used in this study.

Primer	Sequence (5' to 3')	Comments
BamHI linker	CGCGGATCCGCG	5' phosphorylated, self-complementary
EmP280F	CACCACCACCACCACGATGCCTGGGGCATG TTCGTAGC	Forward primer
EmP280R	CAGAGACAGACCACTGCGCATACC	5' phosphorylated, reverse primer
PETF	CGGATCCGAATTCCGCCAACAGTCCCCGGCCAC	Forward primer, contains EcoRI site
PETR	CGGATCCAAGCTTGTAACCACCACACCCGCCGCGC	Reverse primer, contains HindIII site
PIO453F	GGAGGAGGGTCTAGAGCGCCGACATCATAACGGTTCTGGCAAA	Forward primer
MEPOF	GAGCGCGCCTCTAGAAGTCCGTTTAGTGTTCACGA	Forward primer, contains XbaI site
PIO280R	CCGGATGCACAAGCTTGCAGAGACAGACCACTGCGCATACC	Reverse primer, contains HindIII site
MEPOF2	CGCGCAGACTAATTCGAGCTCGGTACCGTCCT	Forward primer
PIO290R	TCCCAAAAGATAAGCTTGCCAGGCATCTCGCCACAACACGTCGCG	Reverse primer, contains HindIII site
PIO300R	TCCCAAAAGATAAGCTTGCCCAAGCAAAGGATGGGCTACGAACAT	Reverse primer, contains HindIII site
PIO310R	TATATAAAGATAAGCTTGCGGCACCGCCGAGAAATGCATGGGCCC	Reverse primer, contains HindIII site
PIO322R	AGTGGTGGGACAAGCTTGCTGCAGCAGCATCTGGTGCGGG	Reverse primer, contains HindIII site
PIO353R	ACCATACCCAAGCTTGTTACGCAGGTAACGCGCGCCGCGAAGCA	Reverse primer, contains HindIII site
PIO376R	ACAAAAGATAAGCTTGGTCCACCTGGGCCAAGACCAGGACCGA	Reverse primer, contains HindIII site
PIO403R	ATTAATACCCAAGCTTGCACGACCGGCTTCGACCAGCGCGCCATGGCG	Reverse primer, contains HindIII site
PIO430R	CCCAAAAGATAAGCTTGCTCGAGCAGCAGCAGCGCCCCAGCAC	Reverse primer, contains HindIII site
PIO440R	TCCCAAAAGATAAGCTTGCTCCTCGGCCGCGGTACGCCCCGGCAC	Reverse primer, contains HindIII site
PIO453R	ACCATACCCAAGCTTGGAACGTGGCGCCTACCGCCGC C	Reverse primer, contains HindIII site
POZ353R	ATTAATATATCCCGGGTTCACGCAGGTAACGCGCGCCGCG	Reverse primer, contains XmaI site
POZ403R	ATTAATATATCCCGGGCACGACCGGCTTCGACCAGCGCGC	Reverse primer, contains XmaI site
281RAF	GCGCAGTGGTCTGTCTCTGGCCGACGTGTTGTGGCGAGATGCC	Forward primer for site-directed mutagenesis
281RAR	GGCTCTCGCCACAACACGTCGGCCAGAGACAGACCACTGCGC	Reverse primer for site-directed mutagenesis
322SCR	TGTACTGGGTAAGCTTTCACTGCAGCAGCATCTGGTGCGGGTGGG CACCGAC	Reverse primer, contains stop codon and HindIII site
430SCR	TGAACGTCGCAAGCTTTCACTCGAGCAGCAGCAGCGCCCCAGCA CGACAATGAC	Reverse primer, contains stop codon and HindIII site
453SCR	CTTGCGTCGCAAGCTTTAGAAACGTGGCGCCTACCGCCGCGTG GATTTTC	Reverse primer, contains stop codon and HindIII site