***pilO*, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin**

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Nucleotide sequencing of a region downstream from the *Pseudomonas aeruginosa* 1244 pilin structural gene, *pilA*, revealed an ORF potentially able to code for a protein of *M*_r 50862. This ORF, called *pilO*, was flanked by a tRNA^{thr} gene, which was followed by a transcriptional termination sequence. The tRNA^{thr} gene and the termination sequence were nearly identical to sequences found immediately adjacent to the *pilA* gene of several *P. aeruginosa* strains. A 2200 base mRNA strand, which contained both the *pilO* and *pilA* transcripts, was produced from this region, while a 650 base transcript containing only *pilA* was present in a 100-fold excess over the longer transcript. Hyperexpression of the *pilA* gene in a *PilO*[−] strain resulted in normal pilus-specific phage sensitivity and twitching motility. The pilin produced by this strain had a lower apparent *M*_r and a more neutral pI compared to that produced by a strain containing a functional *pilO* gene. This pilin failed to react with a sugar-specific reagent which recognized pilin produced by the strain containing a functional *pilO* gene.

Keywords: *Pseudomonas aeruginosa*, type 4 pili, protein glycosylation, pilus assembly, fimbriae

INTRODUCTION

Many Gram-negative bacteria produce thin extracellular protein filaments called pili. The *Pseudomonas aeruginosa* pili are similar in structure and function (Paranchych & Frost, 1988) to those produced by species of the genera *Dichelobacter* (formerly *Bacteroides*; Elleman, 1988), *Eikenella* (Rao & Progulsk-Fox, 1993), *Kingella* (Weir & Marrs, 1992), *Moraxella* (Marrs *et al.*, 1985), *Neisseria* (Meyer *et al.*, 1984) and *Vibrio* (Faast *et al.*, 1989). These pili, which are all organized as bundles of 6-nm-diameter hollow fibres and which have the ability to extend and retract, are classified as type 4 (Ottow, 1975). The pili of *P. aeruginosa* are located at the cell's pole and function to facilitate adherence, a property important in virulence (Baker, 1993; Ramphal *et al.*, 1984; Wood *et al.*, 1980), a form of short-range motility called twitching (Bradley, 1980), and bacteriophage recognition (Bradley, 1972).

The *P. aeruginosa* pili are composed of a 15–16000 *M*_r monomeric pilin subunit, which is arranged in a helical fashion within the pilus fibre (Watts *et al.*, 1983). The first

30 amino acid residues of the pilin from this species are highly conserved, while the remainder vary with strain source. Characteristic variation among pilin primary structure allows differentiation of *P. aeruginosa* pilins into at least two groups (Castric & Deal, 1994). This pilus grouping is further supported by nucleotide sequence homology of the regions flanking the *pilA* gene. The region following the *pilA* gene of *P. aeruginosa* strain 1244 (a group I strain) contains a ribosome-binding sequence and a start codon not seen in the group II strains. This paper presents evidence that this start codon is the beginning of an ORF, referred to as *pilO*. It is proposed that this gene codes for a protein which is involved in post-translational modification of pilin in which an acidic carbohydrate moiety is attached to the pilin protein.

METHODS

Bacterial strains and culture conditions. *P. aeruginosa* 1244, a clinical isolate which has been used in pilus-mediated adhesion studies (Ramphal *et al.*, 1984), was provided by A. T. McManus, US Army Institute of Surgical Research, San Antonio, Texas, USA. *P. aeruginosa* strain 1244N3, a mutant which is unable to make pili or produce pilin (Ramphal *et al.*, 1991) due to an inactivated *rpoN* gene, was furnished by S. Lory, University of

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Washington, USA. Cultures were grown on LB medium (Sambrook *et al.*, 1989) at 37 °C. Antibiotic concentrations in selective media used for *P. aeruginosa* were as follows: carbenicillin (Cb), 500 µg ml⁻¹; tetracycline (Tc), 50 µg ml⁻¹. Antibiotics used with *Escherichia coli* were: ampicillin (Ap), 75 µg ml⁻¹; spectinomycin (Sp), 25 µg ml⁻¹; Tc, 10 µg ml⁻¹.

Nucleotide sequencing. The Sanger dideoxy chain-termination method (Sanger *et al.*, 1977) was used. The template was single-stranded M13 subclones of phage lambda or plasmid clones (Castric *et al.*, 1989) containing DNA downstream from the *pilA* gene. Synthetic primers, derived from known sequences, or universal primers were employed.

Plasmid constructs. The plasmid pPAC46, which contained both *pilA* and *pilO*, was constructed by ligating the *SphI*–*HindIII* plasmid fragment containing *pilA* and adjacent DNA (Fig. 3) of pPAC202 (Castric *et al.*, 1989) to plasmid pUC18 digested with these same enzymes. The *EcoRI*–*HindIII* fragment of this construct, pPAC124, was ligated with the broad host range expression vector pMMB66EH (Ap^r *lacI*^q; Furste *et al.*, 1986), which had been digested with the same restriction enzymes. Plasmid pPAC24, which contained *pilA*, but not *pilO*, was constructed by ligating the pPAC124 *EcoRI*–*NheI* fragment containing the *pilA* gene with pUC19 digested with *EcoRI* and *XbaI*. The *EcoRI*–*HindIII* fragment of this construct, which contained only the *pilA* gene, was ligated with pMMB66EH digested with the same restriction enzymes. The *pilA* gene of both pPAC24 and pPAC46 was under control of the vector *tac* promoter. Since the *SphI* site used in plasmid construction is located within the *pilA* promoter neither pPAC24 nor pPAC46 were able to express the pilin structural gene using the *P. aeruginosa* promoter. Plasmid DNA was introduced into *E. coli* by transformation (Sambrook *et al.*, 1989) and was transferred to *P. aeruginosa* by triparental mating (Ruvkun & Ausubel, 1981).

Northern blot analysis. RNA was extracted as described by Reddy (1989) and separated by formaldehyde-agarose gel electrophoresis, stained with ethidium bromide, and transferred to nitrocellulose paper by capillary blot as described by Sambrook *et al.* (1989). Probe DNA, isolated from pPAC24 or pPAC46, was labelled by nick translation using [α -³²P]dCTP. Prehybridization, hybridization, and washing steps were as described by Sambrook *et al.* (1989). Detection of hybridization was by autoradiography. Densitometric scanning was carried out using an LKB Ultrosan laser densitometer.

Purification of pili. Production of pili by expression of the *pilA* genes of plasmids pPAC24 and pPAC46 in *P. aeruginosa* 1244N3 was carried out using LB-agar- or LB-broth-grown cultures in the presence of 5 mM IPTG. LB-agar-grown cells were resuspended in LB broth. These cells (as well as broth-grown cells) were depiliated by four excursions through a 22-gauge needle. Cells were removed by centrifugation (7500 *g* for 15 min). The pili remaining in the supernatant fluid were purified by a variation of the method of Silipigni-Fusco (1987). In this procedure the supernatant fluid was made 0.1 M with respect to MgCl₂, and stored on ice for 60 min. The resulting precipitate, which contained the pilus fibres, was removed by centrifugation at 12000 *g* for 15 min.

Pilin analysis. N-terminal amino acid sequence analysis and amino acid analysis was performed on purified pilin from strain 1244N3(pPAC24). This protein was subjected to PAGE (15% gel), which was followed by electroblotting to polyvinylidene difluoride paper. Preliminary staining showed that pilin was the major protein present and that it was well separated from the minor contaminants present. Pilin bands were excised and subjected to gas-phase sequencing and to amino acid analysis (University of Pittsburgh Peptide Facility).

Pilin immunoblot analysis. Pilins were separated by PAGE (12.5% gel in the presence of either 0.1% SDS at 5 °C or 30 mM octyl glucoside at 15 °C) or subjected to isoelectric focusing (pH 3.0–9.5) in the presence of 30 mM octyl glucoside at 15 °C using the LKB-Pharmacia Phastsystem. Proteins were transferred to nitrocellulose paper by diffusion blotting; pilin adsorbed to the paper was detected using anti-pilin monoclonal antibody 6–45 (Saiman *et al.*, 1989) as previously described (Castric *et al.*, 1989). The presence of pilin-bound sugar residues was detected by first derivatizing purified pili using the DIG Glycan Detection kit of Boehringer Mannheim Biochemica. This protein was subjected to SDS-PAGE (12.5% gel) using the Bio-Rad Mini-Protein II system, in which temperature was neither controlled nor monitored. Pilin was electroblotted to nitrocellulose paper, probed with an alkaline-phosphatase-labelled antibody specific for the hapten used in pilin derivatization, and developed as described by Boehringer Mannheim Biochemica.

Electron microscopy. Cells to be analysed by electron microscopy were grown on LB agar plates at 37 °C for approximately 18 h and suspended in PBS (0.145 M NaCl, 0.15 M sodium phosphate). The cells were coated on 200 mesh formvar-coated copper grids, subjected to staining with 1% (w/v) uranyl acetate, and viewed in a Philips 201 electron microscope.

Pili functionality tests. The phage sensitivity test was performed by streaking a loop of pilus-specific phage PE69 on an LB agar plate. This was cross-streaked with diluted *P. aeruginosa* 1244 strains to be tested. Phage sensitivity was interpreted from the absence of growth at the cross-streak junction. Twitching (Henrichsen, 1983) was determined by streaking strains of interest on a well-dried LB agar plate where motility was scored as spreading growth at between 48 and 72 h at 37 °C.

RESULTS

Nucleotide sequence analysis

The nucleotide sequence downstream from the *P. aeruginosa* strain 1244 *pilA* gene (Fig. 1) contains a potential transcriptional termination structure situated between positions 626 and 708 (noted previously by Castric *et al.*, 1989). A ribosome-binding site (GGAG) is seen at position 717 followed closely by three start codons, the first of which is located immediately in frame with a stop codon (TGA). The second and third, GTG and ATG, are in frame with each other and begin an ORF (referred to hereafter as *pilO*) which extends 1383 bp, using the ATG codon, to position 2114. Codon usage of the 461 codons of the *pilO* gene is consistent with that of most other *P. aeruginosa* genes (West & Iglewski, 1988). However, significant differences are seen when it is compared with the adjacent *pilA* gene or with the *pilA* genes of other *P. aeruginosa* strains (West & Iglewski, 1988; Castric & Deal, 1994). While the Phe codons, TTT and TTC, have frequencies of 20/29 and 9/29, respectively, in the *pilA* gene, these codons occur at rates of 6/25 and 19/25, respectively, in the *pilO* gene. The Arg codons, CGT and GCG, appear at rates of 26/31 and 2/31 in the *pilA* gene, but occur at rates of 7/31 and 11/31, respectively, in the *pilO* gene. In addition, the G+C% content of *pilO* (60.1%) is consistent with the general range given for *P. aeruginosa* chromosomal DNA (West & Iglewski, 1988), but quite different from the value of 52.2% determined for the strain 1244 *pilA* gene. A search of the GenBank

1249

1 kb

S N P H 1244

pilA *pilO* tRNA

PA103

pilA tRNA

1250

Fig. 4. Northern blot analysis of mRNA extracted from *P. aeruginosa* strain 1244. (a) Probes were prepared from restriction fragments of cloned DNA indicated in the Fig. 3 map as follows. Lanes: 1, probe 1 (the *SphI*-*NheI* fragment); 2, probe 2 (the *NheI*-*PstI* fragment); 3, probe 3 (the *PstI*-*HindIII* fragment). Arrows indicate positions of *E. coli* strain HB101 ribosomal RNA. (b) Densitometric scan of lane 1 of part (a) of this figure. Scanning was from bottom to top of the autoradiogram shown. The electrophoresis point of origin is marked by the arrow.

expression of the *pilA* gene, carried on recombinant plasmids pPAC24 (containing only *pilA*) or pPAC46 (bearing both *pilA* and *pilO*), under control of a *tac* promoter. Electron microscopy showed that while strain 1244N3 produced no pili both derivatives 1244N3(pPAC24) and 1244N3(pPAC46), when grown in the presence of IPTG, produced pilus fibres which were indistinguishable from those of the wild-type (results not

Fig. 5. Immunodetection of electrophoretically separated *P. aeruginosa* pilin. (a) SDS-PAGE of pilin at 5 °C. Lanes: 1, 3 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC46); 2, 3 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC24). M_r values are indicated on the left. (b) Isoelectric focusing of pilin at 15 °C. Lanes: 1, 3 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC24); 2, 3 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC46). The pH gradient was determined by focusing pI standards on an identical gel in the absence of octyl glucoside.

shown). Both strains 1244N3(pPAC24) and 1244N3(pPAC46), in the presence of IPTG, were sensitive to the pilus-specific bacteriophage PE69 and demonstrated twitching motility on dried agar plates, properties lacking in strain 1244N3. These results indicated that the absence of *pilO* did not appear to influence the ability of the pilus to extend and retract, qualities necessary for twitching and for phage sensitivity.

Influence of *pilO* on pilin structure

Although strain 1244N3 was unable to produce pilin, as determined by Western blot analysis (results not shown), complementation of this strain with either plasmid pPAC24 or pPAC46, under inducing conditions, allowed production of this protein (Fig. 5a). While the M_r of mature pilin of strain 1244 predicted from the nucleotide sequence of the *pilA* gene (Castric *et al.*, 1989) is 15653, the apparent size of pilin produced by strain 1244N3(pPAC46) or by wild-type strain 1244 (results not shown) is approximately 16900. Pilin produced by strain 1244N3(pPAC24), which lacks a functional *pilO* gene, had an apparent M_r of 15600. Identical results were obtained when gradient SDS-PAGE was employed. A small fraction of the pilin produced by strain 1244N3(pPAC24) migrated with wild-type pilin when the Bio-Rad Mini-Protein II PAGE system (15 % gel) was employed (results not shown).

To explore the significance of this apparent M_r difference it was necessary to see if the N-terminus of pilin produced

Fig. 6. Detection of *P. aeruginosa* pilin carbohydrate by immunoblot. Lanes: 1, 10 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC46); 2, 10 µg pilin produced by *P. aeruginosa* strain 1244N3(pPAC24). M_r values are indicated on the left. Arrows indicate positions of these pilins on an identical blot using monoclonal antibody 6-45 for detection.

by strain 1244N3(pPAC24) was in any way altered. When this protein was subjected to N-terminal amino acid analysis, the sequence of mature pilin, XTLIELM (the first residue produced no reaction presumably because it was the masked amino acid *N*-methyl-phenylalanine), was obtained. Amino acid analysis of blotted pilin was also carried out to determine the integrity of the carboxy-terminal region. The pilin of *P. aeruginosa* 1244 contains six prolines (Castric *et al.*, 1989), mainly in the carboxy-terminal region. The observed M_r difference of approximately 1200, if caused by cleavage in this region, would require breaking the bond between residues 136 and 137 of mature pilin resulting in a change in apparent proline number from six to three. Normalization (with alanine) of actual values obtained gave a proline/alanine (P/A) ratio of 0.36, with a P/A ratio of 0.20 predicted for a truncated form and a P/A ratio of 0.35 predicted for mature pilin. Normalization with leucine values gave a proline/leucine (P/L) ratio of 0.68 (a P/L ratio of 0.33 is predicted for a truncated form and a P/L ratio of 0.67 for mature pilin). These results suggest that the pilin carboxy-terminal region must be substantially intact.

Purified pili from strains 1244N3(pPAC24) and 1244N3(pPAC46) were treated with periodic acid to oxidize any sugar residues present. These proteins were then treated with digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide which will form a covalent bond with oxidized sugars. After SDS-PAGE these proteins were electroblotted to nitrocellulose paper and probed with an anti-digoxigenin antibody preparation. Fig. 6 shows that pilin from strain 1244N3(pPAC46) reacted with this antibody indicating the presence of sugar moieties, while pilin from strain 1244N3(pPAC24) gave no reaction.

Pili were dissociated into pilin monomers and dimers in the presence of 30 mM octyl glucoside (Watts *et al.*, 1982a, b, 1983), subjected to isoelectric focusing, then blotted to nitrocellulose paper and probed with pilin-specific monoclonal antibodies. Pilin produced by strain 1244N3(pPAC46) or by wild-type strain 1244 (results not shown) had a pI of 4.75 (Fig. 5b). Pilin from strain 1244N3(pPAC24), which lacked a functional *pilO* gene,

had a pI of approximately 6.25. The pI of mature strain 1244 pilin is predicted from the *pilA* gene sequence (assuming that the N-terminus is blocked and the two cysteines form a disulfide) to be 7.00. These results indicate that a significant alteration in pilin charge arrangement (neutralization of positive charges or introduction of negative ones) is brought about by the presence of the *pilO* gene. The difference in charge between these two pilin forms was confirmed by PAGE in the presence of 30 mM octyl glucoside at pH 8.8. Pilin from strain 1244 or strain 1244N3(pPAC46) had a greater net negative charge than pilin from strain 1244N3(pPAC24) (results not shown). Pilin from strains 1244 and 1244N3(pPAC46) also focused at pH 4.75 in the presence of 8.0 M urea and 2.5% (v/v) Triton X-100 (results not shown). Pilin preparations from strain 1244N3(pPAC46) or the wild-type strain contained trace amounts of the neutral pilin form (results not shown) possibly due to chemical or enzymic action. The small amounts of the acidic pilin form produced by strain 1244N3(pPAC24) (Fig. 5b), as well as the higher M_r form described above, may be due to low levels of *pilO* gene expression by the host strain. These results altogether suggest that the *P. aeruginosa* strain 1244 *pilO* gene is required for a post-translational pilin modification, specifically a glycosylation.

DISCUSSION

While glycosylation of prokaryotic proteins appears to be primarily restricted to the archaeobacteria (Lechner & Wieland, 1989), reports have suggested the association of polysaccharide with pili (Armstrong *et al.*, 1981; Robertson *et al.*, 1977). Virji *et al.* (1993) presented strong evidence (carbohydrate detection of blotted pilin and chemical removal of sugars) that *Neisseria meningitidis* pilin is glycosylated. Results presented in this paper suggest that pilin from *P. aeruginosa* strain 1244 also is glycosylated. The composition of the pilin-associated material remains to be determined. However, several candidates, including the acidic moieties of the lipopolysaccharide core, alginic acid subunits, as well as other acidic elements or phosphorylated compounds, must be considered. Limitations of SDS-PAGE prevent reliable M_r determinations of glycosylated proteins, a test which must await the use of a more accurate technique such as mass spectrometry. Linkage of glycosylated proteins is usually via the reducing end of an oligosaccharide sugar through an O- or N-linkage to the hydroxyl group of serine (or threonine) or the amide group of asparagine (Montreuil *et al.*, 1986). The residues in the region of N-linked moieties have the characteristic consensus sequence N-X-S (or T). While this sequence may be found in the *N. meningitidis* pilin primary structure (Virji *et al.*, 1993), it is absent in *P. aeruginosa* strain 1244 pilin, suggesting the presence of an N-linkage, a different sequence specificity, or the utilization of an alternative method of attachment. Clearly much work remains in the characterization of this pilin-associated material.

Previous work (Frost & Paranchych, 1977; Paranchych *et al.*, 1979) has indicated that pili from *P. aeruginosa* strains

PAO and PAK contained no sugar residues. Since the primary structure of pili from strain 1244 is distinctive when compared to those of strains PAO and PAK (Castric & Deal, 1994), pilus modification may represent a strain difference. *P. aeruginosa* strains producing pili antigenically related to those of strain 1244 are common among clinical isolates (Castric & Deal, 1994). Thus, pilin glycosylation by this bacterium could be useful in clinical identification. Likewise, demonstration of the presence of the *pilO* gene could be of diagnostic value.

Since the absence of glycosylation seems to have no effect on either the formation of pilus fibres or extension and retraction of these fibres (as measured by bacteriophage sensitivity and twitching motility), the function of this modification is unclear. Because all pilin monomers appear to be modified [this must include both the pilin membrane pool (Watts *et al.*, 1982c) as well as pilus fibres], these glycoproteins must contribute greatly to the overall cell surface negativity. Bacterial avoidance of phagocytosis through inhibition of attachment has been well documented (Densen & Mandell, 1980). Diminished phagocytosis by neutrophils because of electrostatic repulsion has been demonstrated both by streptococcal M protein (Fischetti, 1989) and the pili of *Neisseria gonorrhoeae* (Heckels *et al.*, 1976). Such a mechanism could also benefit *P. aeruginosa* when growing saprophytically, where the avoidance of phagocytic amoebae could be an important survival mechanism.

Specific recognition by the pili allows attachment of the pathogen to host cells containing the proper receptors (Baker, 1993; Heckels *et al.*, 1976). The acidic modification might stabilize this attachment through the formation of salt bridges once specific binding had occurred. Alternatively pilin glycosylation may be involved in specific or nonspecific adhesion to the host cell which is independent of pilin protein-mediated attachment. Adhesion studies utilizing PilO⁺ and PilO⁻ variants of *P. aeruginosa* strain 1244, as well as glycosylated and nonglycosylated pili, will be required to clarify these points.

The genetic evidence presented in this paper relating the presence of pilin glycosylation to a functioning *pilO* gene indicates that this process has a specific cellular role. This is strongly supported by the findings that the *pilO* gene is part of the *pilA* transcriptional unit. Although the *pilA* and *pilO* genes, along with a tRNA^{thr} gene, are present in the form of an operon, two major transcriptional products are generated (an individual *pilA* message and a *pilA/pilO/tRNA^{thr}* transcript) which are present in a ratio of about 100:1. Such results would not be unexpected as pilin is a major cell protein, while PilO would likely be required in only catalytic or regulatory amounts. The difference in transcription levels could be the result of premature termination of message synthesis or relative instability of the transcript corresponding to the *pilO* region. The role of translation efficiency should also be considered a factor in differential gene expression since the beginning of the *pilO* message contains a short loop region which includes the start codon. Such a structure has been suggested to be able to significantly reduce

translation (Gold, 1988), and may function to further control expression of a gene coding for a product required in small amounts when it shares a promoter with a gene coding for a product needed in large amounts.

The *pilO* gene product is predicted to code for a protein with a *M_r* of 50862 using as start codon the ATG beginning at position 729 (Fig. 1). Hydropathy profile (Kyte & Doolittle, 1982) of the primary structure indicates that PilO contains nine hydrophobic regions which are flanked by clusters of charged residues. Secondary structure prediction (Chou & Fassman, 1974) suggests that large portions of these hydrophobic regions are composed of β -structure which are of adequate length to span the 3.0 nm membrane lipid core. Positively charged residues flanking the hydrophobic segments could be expected to stabilize this structure through ionic interaction with membrane phospholipids, while charged and polar residues in these regions would promote a surface solvent interaction on both sides of a membrane. Although the location of PilO has not been demonstrated, the high degree of hydrophobicity within the predicted transmembrane regions suggests that it resides in the cytoplasmic membrane. This location would be ideal if PilO functions catalytically on the periplasm side of the cytoplasmic membrane to transfer carrier lipid-bound oligosaccharide subunits to emerging pilin monomers.

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