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Identification of a Novel Gene, *pilZ*, Essential for Type 4 Fimbrial Biogenesis in *Pseudomonas aeruginosa*

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The opportunistic pathogen *Pseudomonas aeruginosa* produces type 4 fimbriae which promote adhesion to epithelial cells and are associated with a form of surface translocation called twitching motility. We have used transposon mutagenesis to identify loci required for fimbrial assembly or function by screening for mutants that lack the spreading colony morphology characteristic of twitching motility. A subset of these mutants is resistant to fimbria-specific phage. One of these mutants (R270) was found to contain a transposon insertion in a new gene, termed *pilZ*, which is located on chromosomal *Spe*I fragment I at about 40 min on the *P. aeruginosa* map, a position remote from other loci involved in fimbrial biogenesis. *pilZ* appears to be linked to and possibly forms an operon with a gene, *holB, which is homologous to the gene encoding the δ′ subunit of *Escherichia coli* DNA polymerase III. The product of the *pilZ* gene is a protein of 118 amino acids (predicted molecular weight, 12,895) which probably has a cytoplasmic location. PilZ appears to be a new class of protein which has not hitherto been represented in the sequence databases, and its function is unknown. Complementation studies indicate that *pilZ* is able to restore the expression of fimbriae on the surface of *P. aeruginosa*, as well as twitching motility and sensitivity to fimbria-specific phage when provided in *trans* to the R270 mutant.**

Type 4 fimbriae (or common pili) are flexible, filamentous surface appendages which are found in a number of important pathogenic bacteria, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella bovis*, *Dichelobacter nodosus*, and *Pseudomonas aeruginosa*, and which mediate adherence to host epithelial tissue during colonization (33, 40, 42). Type 4 fimbriae were originally defined by their polar arrangement on the cell and their ability to promote a form of motion called twitching motility (14, 32). Type 4 fimbriae are also characterized by conserved features of the structural subunit proteins which make up the fimbrial strand. These include a short, positively charged leader sequence of six to eight amino acids which is removed during fimbrial assembly, a modified amino acid, *N*-methylphenylalanine, as the first residue in the mature protein, and a highly conserved hydrophobic amino-terminal domain (7, 42).

The biogenesis of type 4 fimbriae is a complex process involving a large network of genes that have thus far been localized to three separate regions of the *Pseudomonas* chromosome. The largest locus is located on *Spe*I fragment E at about 70 min on the physical map and contains the fimbrial subunit gene (*pilA*), two ancillary genes (*pilB* and *pilC*), the specific leader peptidase gene (*pilD*), and the sensor/regulator genes (*pilS* and *pilR*) responsible for the transcriptional activation of the fimbrial subunit gene (16, 21, 41). Recently, two further genes (*pilE* and *pilV*) have been localized to this region of the genome, both of which encode proteins possessing prepilin-like leader sequences that appear to be cleaved by the PilD peptidase (2, 35). A second group of fimbria-associated genes is found on *Spe*I fragment H at ~20 min on the physical map. These include the *pilT* and *pilU* genes which encode two potential nucleotide-binding proteins involved in fimbrial function, since mutations in either gene cause loss of twitching motility and apparent hyperfimbriation (47, 48). About 10 kb from *pilT* and *pilU* is a cluster of genes (*pilGHIJK*) which encode a set of proteins with close resemblance to members of the enteric chemotactic network, which suggests a related signal transduction system that regulates fimbrial function, possibly the directional control of twitching motility (8–10). The third fimbria-associated region is found on *Spe*I fragment B at ~2 min on the physical map and contains an operon of five genes, *pilMNOPQ*, all of which are required for fimbrial assembly (25, 26).

The infrastructure and machinery required to produce and assemble type 4 fimbriae appear to be well conserved among the different bacterial species that possess these structures. This is indicated by the observation that *P. aeruginosa* is capable of producing fimbriae composed of heterologous subunits expressed from cloned subunit genes from *D. nodosus*, *M. bovis*, and *N. gonorrhoeae* (3, 19, 27). A number of homologs to the fimbria-associated genes of *P. aeruginosa* have been identified in other type 4 fimbriate bacteria (20, 24). However, it is also clear that a number of genes involved in the biogenesis and function of type 4 fimbriae remain unidentified. In this paper, analysis of a bacteriophage-resistant fimbrial mutant has enabled identification and characterization of a novel protein that is essential for correct fimbrial export in *P. aeruginosa.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *P. aeruginosa* mutant R270 is a derivative of wild-type strain PAK (David Bradley, Memorial University of Newfoundland, St. Johns, Newfoundland, Canada) and constitutes part of the transposon mutant collection held in our laboratory. The *Escherichia coli* strain XL-1 Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* [F′, $proA^+B^+$ $lacI^q$ *lacZDM15* Tn*10*(tet^r)]) was used as a host for all manipulations including generation of single-stranded DNA templates prior to DNA sequencing. The DNA inserts of recombinant plasmids used throughout this study are shown in Fig. 1, and were contained in either pBluescript (Stratagene) or in the shuttle vectors pUCP18/19 (kindly provided by H. Schweizer) which replicate stably in both *Pseudomonas* and *E. coli* hosts (38). Bacteriophage PO4 is a PAK fimbria-specific bacteriophage described elsewhere (5), and sensitivity to infection by this bac-

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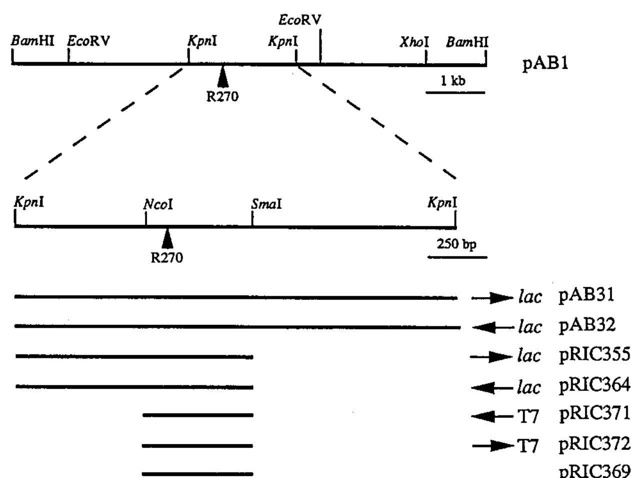

FIG. 1. Schematic representation of pAB1 and the R270 fimbrial gene locus. The relevant restriction sites are shown, and the site of transposon insertion is indicated by an arrowhead. The relevant plasmid inserts are shown diagrammatically, and their orientations with respect to the external *lac* or T7 promoters are indicated if relevant.

teriophage was determined quantitatively as described elsewhere (2). The PAO cosmid library was generously provided by B. Holloway (Monash University, Clayton, Victoria, Australia). All bacterial strains were maintained on LB medium (36) containing 100 mg of ampicillin (*E. coli*) or 500 mg of carbenicillin (*P. aeruginosa*) per ml if maintenance of plasmids was required.

Recombinant DNA techniques and transformation. Preparation of plasmid DNA, digestion with restriction endonucleases (New England Biolabs, Beverly, Mass.), ligations, and transformation in *E. coli* hosts were carried out under standard conditions as described by Sambrook et al. (36). Preparation of *P. aeruginosa* competent cells and transformation were achieved as described previously (2).

Subsurface twitching assay. The quantitative assay for twitching motility was an adaptation of previous methods described by McMichael (28), with modifications described elsewhere (2), except that the agar was not removed after staining.

Protein expression. Plasmid-encoded proteins were visualized by subcloning DNA fragments of interest into the T7 expression vector pEB15 (6). These constructs were then transformed into *P. aeruginosa* ADD1976 which contains a T7 RNA polymerase gene under *lac* control introduced into the chromosome (6). Induction and specific labelling of proteins under T7 control were performed as described previously (2).

Sequencing and protein analysis. A series of subclones and deletions were constructed for DNA sequence analysis, and single-stranded DNA sequencing was achieved by the dideoxy chain termination method (37) with the Pharmacia T7 deazaG/A sequencing kit. Sequence analysis was performed with the MacVector 4.0.1 software program. The deduced protein sequences were analyzed with the Genetics Computer Group (University of Wisconsin, Madison) software packages (11). TFASTA and BLAST searches to the GenBank data bases were performed through the Australian National Genome Information Service.

Fimbrial subunit preparation and immunoblotting. Surface fimbrial preparations were made as described elsewhere (2). Whole-cell protein samples were prepared by suspending fresh overnight growth in 1× sample buffer. All samples were then heated to 100°C for 5 min prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel (Bio-Rad) by the method described by Laemmli (23). The proteins were then transferred electrophoretically to Hybond-C nitrocellulose paper (Amersham) in the Tris-glycine buffer system described by Towbin et al. (44) and were detected with antifimbrial antiserum generated against the *P. aeruginosa* PAK PilA protein (1:5,000) and goat-anti rabbit immunoglobulin G conjugated to alkaline phosphatase (1:5,000).

Cell fractionation. After removal of surface fimbrial filaments as described above, the bacterial cells were fractionated as follows. Periplasmic contents were released following the $MgCl_2$ treatment method described by Hoshino and Kageyama (18), and the cells were further separated into membrane and cytoplasmic fractions as described by Hancock and Nikaido (13).

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF-DR II contour-clamped homogeneous electric field system (Bio-Rad). The preparation and digestion of *P. aeruginosa* PAO DNA plugs were essentially as described previously by Ratnaningsih et al. (34). Electrophoresis was performed through a 1.5% agarose gel at 170 V at 14°C. The pulse times used were 30 s for 12 h and then 60 s for 52 h. After electrophoresis, the PFGE-separated DNA was transferred to nylon membrane (Hybond N; Amersham) and probed with the 1.8-kb insert from pAB6 by standard procedures (36).

Preparation of RNA and Northern (RNA) blotting. Total RNA was extracted from *P. aeruginosa* strains by the method described previously (16). RNA was electrophoresed under denaturing conditions and transferred to nylon membrane (Hybond N; Amersham) as described elsewhere (16). The probe used was the purified insert contained within plasmid pRIC369 labelled with [^{32}P]dCTP, and hybridizations and washes were performed according to the protocol of the manufacturer.

RESULTS

Identification of the *pil*-specific mutant and cloning of the affected locus. To genetically define all of the components involved in the biogenesis of type 4 fimbriae in *P. aeruginosa*, the wild-type strain PAK was subjected to transposon mutagenesis with Tn*5*-B21 (16). The initial screening method was to identify mutants that had become deficient in twitching motility, a phenotype that requires the presence of functional fimbriae. Such mutants exhibit an altered colony morphology which appears smooth and domed, in contrast to the rough-edged spreading colony morphology of wild-type *Pseudomonas* cells. These mutants were then classified according to their sensitivity or resistance to infection by the fimbria-dependent bacteriophage PO4. Hybridization analyses of the 28 bacteriophage resistant mutants with the transposon grouped these mutants into six categories based on the size of the *Bgl*II restriction fragment into which the transposon had inserted (16). One of these groups, R6, contained eight mutants which contained large hybridizing *Bgl*II fragments and which were likely to be heterogeneous. This has proved to be the case, and two of these mutants have been recently localized to the *pilV* gene (2).

The flanking chromosomal DNA from mutant R270 (a member of the R6 group) was cloned by marker rescue, i.e., digestion of the genomic DNA, ligation into pBluescriptSK$^+$, and subsequent selection for the tetracycline resistance gene encoded on Tn*5*-B21. This plasmid, pR270, was used to probe a wild-type PAO1 cosmid library to obtain an intact copy of the affected locus. After subcloning of the cosmid, the location of transposon insertion was localized to an 8-kb *Bam*HI fragment, which was designated pAB1 (Fig. 1). The pAB1 plasmid was then used to probe Southern blots from the remaining members of the R6 group to determine if any of the other mutants in this group had been disrupted at the same locus. All other members of the R6 mutant group had unchanged *Bam*HI fragments, indicating that R270 was a single mutation at this locus in the transposon library. Southern analysis on plasmid pAB1 further localized the site of transposon insertion in R270 to a *Kpn*I fragment of approximately 1.8 kb (Fig. 1).

The ability of mutant R270 to produce the PilA fimbrial subunit and the stage at which the fimbrial biogenesis process had been disrupted were investigated by separating the cellular compartments and localizing PilA by immunoblotting (Fig. 2). Fractionation of the wild-type strain PAK and the R270 mutant identified a large intracellular pool of processed PilA subunits in the membrane fraction (Fig. 2, lanes 3 and 7), with little or no detectable protein in either the periplasmic or

FIG. 2. Immunoblot of the PilA fimbrial subunit found in cellular fractions of *P. aeruginosa* PAK (lanes 1 to 4) and R270 (lanes 5 to 8). The cell fractions are extracellular shear fraction (lanes 1 and 5), periplasmic fraction (lanes 2 and 6), membrane fraction (lanes 3 and 7), and cytoplasmic fraction (lanes 4 and 8). The PilA subunit is indicated by an arrow.

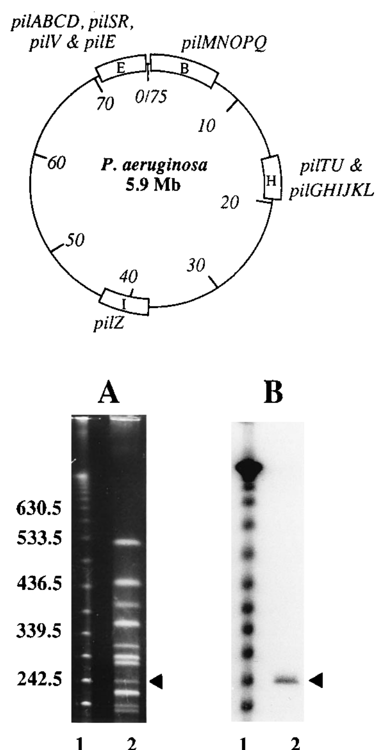

FIG. 3. Physical mapping of the R270 mutation. The top panel represents a schematic map of the *P. aeruginosa* PAO1 chromosome showing positions of the currently known fimbria-associated genes. The gradations refer to the 75-min genetic map described by Ratnaningsih et al. (34). (A) *Spe*I-digested chromosomal DNA from *P. aeruginosa* PAO1, electrophoresed on a pulsed-field gel and stained with ethidium bromide. The size markers (in kilobases) shown on the left are concatamerized lambda phage genomes (New England Biolabs), and the *Spe*I fragment I, originally estimated to be 290 kb (34), migrates alongside the 242.5-kb marker and is indicated by an arrowhead. (B) Southern blot of the gel shown in panel A showing specific hybridization of *Spe*I fragment I (arrowed) to a probe of the R270 locus (pAB31).

cytoplasmic fractions (Fig. 2, lanes 2, 4, 6, and 8). The wild-type strain also contained a large amount of PilA in the shear fraction which represents surface-located fimbrial subunits (Fig. 2, lane 1), whereas the surface of the R270 mutant was devoid of PilA protein (Fig. 2, lane 5). The lack of assembled surface fimbrial filaments on R270 was also confirmed by electron microscopy (data not shown). These data suggest that the PilA subunits are accumulating within the membrane of the R270 mutant and are unable to be assembled or exported to the cell surface.

Physical mapping of the fimbrial locus. The locus represented by mutant R270 was mapped on the *P. aeruginosa* PAO1 genome by using the *Spe*I map of Ratnaningsih et al. (34). *Spe*I-digested PAO1 DNA was separated by PFGE, transferred to a nylon membrane, and probed with the 1.8-kb *Kpn*I fragment from pAB31 (Fig. 1), which had been shown by Southern analysis to contain the site of transposon insertion. The probe hybridized to a 260-kb fragment that represents *Spe*I fragment I and which is located at approximately 40 min on the genome (Fig. 3). This locus is quite separate from the other three regions of the chromosome shown to contain fimbria-associated genes: *Spe*I fragments E (*pilA-D pilSR pilV pilE*), H (*pilTU pilG-K*) and B (*pilM-Q*) (Fig. 3A). The region at 40 min of the *P. aeruginosa* genome has previously been shown to contain the *xcp* genes that are responsible for extracellular protein export (39). The two systems of extracellular protein export and fimbrial biogenesis have many parallels (2, 17, 26a), with a number of their members displaying significant amino acid homology and the PilD endoprotease playing an important role in both systems, since it is responsible for cleavage and methylation of both pre-PilA and pre-XcpT-W. To determine whether the locus affected in mutant R270 was fimbria specific or, like *pilD*, involved in both systems, the status of extracellular protein export was investigated. The secretion of both elastase and alkaline phosphatase was assayed in the R270 mutant and was found to be identical to the parental PAK strain (data not shown), suggesting that the affected locus is specific to the type 4 fimbrial biogenesis pathway.

DNA sequence and analysis of the affected locus. The entire insert in plasmid pAB31 and some flanking DNA were sequenced, and the sequence is presented in Fig. 4. This 2.0-kb fragment has a G+C content of 66%, which is in excellent agreement with the value of 67% that has been estimated for the *Pseudomonas* genome (46). This fragment contained three open reading frames (ORFs) that possessed the high bias for G+C residues in the third position of the coding triplet that is characteristic of *Pseudomonas* genes (46). Two ORFs (ORF1 and ORF2) were present on the plus strand in a putative operonic structure because of the close proximity of their initiation and termination codons and the lack of a recognizable transcriptional termination signal between them (Fig. 4) (29). These ORFs possessed third-position G+C content values of 84 and 89%, respectively. The third ORF, ORF3, was found downstream of ORF2 in the opposite direction on the minus strand and had a third-position G+C content value of 81%. The termination codons of ORF2 and ORF3 are separated by 135 bp, within which is a large inverted repeat with a free energy value of −25 kcal (ca. −105 kJ)/mol, which may function as a bidirectional transcriptional terminator (Fig. 4). The nucleotide sequence obtained with an oligonucleotide directed at the inverted repeat of Tn*5*-B21 from plasmid pR270 localized the point of transposon insertion in mutant R270 at position 1319, which would therefore selectively disrupt ORF2 (Fig. 4). The predicted product of ORF2 is 118 amino acids in length, with a predicted molecular weight of 12,895, and that of ORF3 is 114 amino acids, with a predicted molecular weight of 12,944. The three translated ORFs were used to perform BLAST and TFASTA searches on all available DNA and protein sequence databases. Although no significant homologies were detected with ORF2 and ORF3, ORF1 displayed strong homology over its entire length with the *E. coli holB* gene (29% identity and 49% similarity) which encodes the δ' subunit of DNA polymerase III (Fig. 5). Although we have no direct biochemical evidence to support the function of ORF1, the predicted protein sequence contains the motifs that are conserved between the δ', γ, and τ proteins of the DNA polymerase III holoenzyme (Fig. 5). This ORF, designated *holB**, represents only the second prokaryotic δ' subunit gene to be sequenced. It is unlikely that ORF3 is incomplete and extends further than shown, since the third-position G+C content in the upstream region (in the same reading frame) drops dramatically to 61%.

Complementation of the R270 *pil* mutant. From nucleotide sequence analysis of the surrounding region, it appeared that the 1.77-kb *Kpn*I fragment contained the entire ORF that was disrupted by the transposon in mutant R270. Furthermore, since this ORF was directly followed by a potential transcriptional termination loop, it was unlikely that the transposon insertion would exert any polar effects on the expression of distal genes.

Therefore, the 1.77-kb *Kpn*I fragment was examined for its

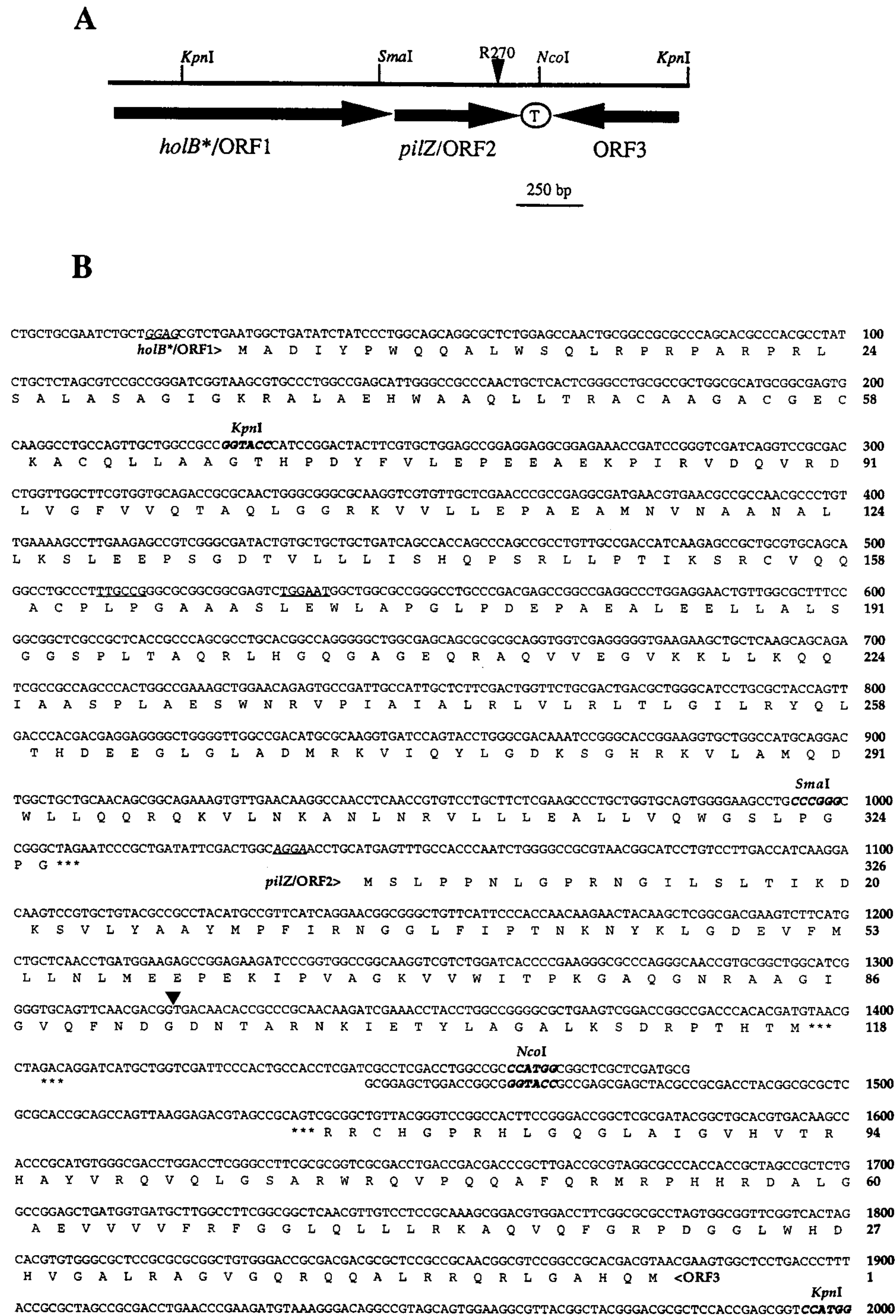

FIG. 4. (A) Schematic representation of the ORFs located around the R270 transposon insertion site. A bidirectional transcriptional terminator is indicated by T. The transcriptional orientations of the genes are indicated by arrows. (B) The nucleotide sequences and predicted amino acid sequences of the ORFs. Only the coding strand for each ORF is shown, and important restriction enzyme sites are indicated. The location of the transposon insertion point for mutant R270 is indicated by an arrowhead. The bidirectional transcriptional terminator is shown as double stranded. Potential promoter sequences for *pilZ* are underlined, and putative ribosome-binding sites for *holB** and *pilZ* are underlined and italicized. This sequence has been deposited in GenBank under accession number L42622.

ability to complement the R270 mutant, with the restoration of twitching motility and phage sensitivity used as phenotypic tests, in mutant R270 containing pAB31, pAB32, and pUCP19 as the control. The R270[pUCP19] strain lacked twitching motility (Fig. 6B), whereas both R270[pAB31] and R270[pAB32] complemented the twitching phenotype (Fig. 6C and D) back to wild-type levels (Fig. 6A). Both the pAB31 and pAB32 plasmids were also able to revert R270 back to wild-type levels of bacteriophage infection ($\sim 10^{11}$). Since both pAB31 and pAB32, which contained the 1.77-kb *Kpn*I fragment in both

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HolBE.c   -MRWYPWLRPDFEKLVASYQAGRGHHALLIQALPGMGDDALIYALSRYLLCQQPQGHKSCGHCRGCQLMQAGTHPDYYTLAPEKGKNTLG  89
HolB*P.a  MADIYPWQQALWSQL--RPRPARPR--LSALASAGIGKRALAEHWAAQLLTR-ACAAGACGECKACQLLAAGTHPDYFVLEPEEAEKPIR  85
              *** .     .*     .  * .  *   *  *.*  **     .  **..       .** *. ***. *******. * **. .  .

HolBE.c   VDAVREVTEKLNEHARLGGAKVVWVTDAALLTDAAANALLKTLEEPPAETWFFLATREPERLLATLRSRCRLHYLAPPPEQYAVTWLSRE  179
HolB*P.a  VDQVRDLVGFVVQTAQLGGRKVVLLEPAEAMNVNAANALLKSLEEPSGDTVLLLISHQPSRLLPTIKSRCVQQACPLPGAAASLEWLAPG  175
          ** **..   . . *.*** *** .  *   ..  *******.****  .*   *  ...* *** *..***  .     *    .. **.

HolBE.c   VTMS-QDALLAALRLSAGSPGAALALFQGDNWQARETLCQALAYSVPSGDWYSLLAALNHEQAPARLHWLATLLMDALKRH---HGAAQV  265
HolB*P.a  LPDEPAEALEELLALSGGSPLTAQRLHGQGAGEQRAQVVEGVKKLLKQQIAASPLAESWNRVPIAIALRLVLRLTLGILRYQLTHDEEGL  265
          .    .**   * ** *** .*  *        . *  . . .   .        * **   .    *    *    *   . *    *   .

HolBE.c   TNVDVPGLVAELANHLSPSRLQAILGDVCHIREQLMSVTGINRELLITDLLLRIEHYLQPGVVLPVPHL  334
HolB*P.a  GLADMRKVIQYLGDKSGHRKVLAMQDWLLQQRQKVLNKANLNRVLLLEALLVQWGSLPGPG         326
             *.  ..  *  .    .. *.   . . *..... ...** **.  **..      **
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FIG. 5. Sequence alignment of the *P. aeruginosa* (P.a.) *holB** gene with the *holB* gene from *E. coli* (E.c.). The alignments were generated with the ClustalV program (15). Identical residues are indicated by asterisks, while conserved residues are indicated by dots. The highly conserved areas between the *E. coli* γ, τ, and δ′ subunit proteins are overlined.

directions with respect to the external *lac* promoter (Fig. 1), were able to fully complement R270 back to the wild-type phenotype, it is likely that this fragment not only contained the gene of interest, but also its native transcriptional promoter.

These results were confirmed by immunoblotting both whole-cell and surface protein preparations from these strains (Fig. 7A and B). Analysis of the whole-cell samples indicates that PilA production was unaltered in R270 carrying any of the plasmids listed above (Fig. 7A, lanes 2 to 4), compared with the PAK control strain (Fig. 7A, lane 1). The surface preparation of R270[pUCP19] contained no PilA protein as expected (Fig. 7B, lane 2), whereas both R270[pAB31] and R270[pAB32] produced PilA on the cell surface (Fig. 7B, lanes 3 and 4) to levels comparable to that of the PAK wild-type strain (Fig. 7B, lane 1).

The 1-kb *Sma*I-*Kpn*I fragment (Fig. 1 and 4) that contained the entire ORF2 and ORF3 (separated by the transcriptional terminator) was then subcloned in both orientations with respect to the *lac* promoter and then tested for its ability to complement the R270 mutant. When the phenotypes of R270 [pRIC364], containing ORF2 driven by the *lac* promoter, were analyzed, it was found that this strain produced PilA on the cell surface (Fig. 7B, lane 6), was able to undergo twitching motility (Fig. 6F), and was fully sensitive to bacteriophage PO4 infection to wild-type levels. In contrast, R270[pRIC355], containing ORF2 against the *lac* promoter, failed to produce PilA on the cell surface (Fig. 7B, lane 5), was nontwitching (Fig. 6E), and maintained its total resistance to bacteriophage PO4 infection. These results suggest that although ORF2 is sufficient to revert mutant R270 to wild-type phenotypes, its transcriptional promoter must lie upstream of the *Sma*I restriction site. The ORF2 gene was designated *pilZ*.

FIG. 6. The subagar surface translocation assay used to determine twitching motility. Zones of motile *P. aeruginosa* cells are as follows: A, PAK; B, R270 [pUCP19]; C, R270[pAB31]; D, R270[pAB32]; E, R270[pRIC355]; F, R270 [pRIC364].

Expression of *pilZ* in *P. aeruginosa*. The *pilZ* gene was cloned into the broad-host-range T7 expression vector pEB15. Plasmid pRIC371 contained the *pilZ* gene as the *Sma*I-*Nco*I fragment in the correct orientation with respect to the T7 promoter, whereas plasmid pRIC372 contained the insert in the opposite orientation. Both plasmids were transformed into the *P. aeruginosa* strain ADD1976 that contains the T7 DNA polymerase gene under *lac* control stably inserted into the chromosome. After induction with isopropyl-β-D-thiogalactopyranoside and specific labelling with [^{35}S]methionine, the *P. aeruginosa* strain ADD1976 carrying plasmid pRIC371 produced a unique protein with an apparent molecular mass of 13 kDa, corresponding to that of the PilZ protein (Fig. 8, lane 2), compared with plasmid pRIC372 (Fig. 8, lane 3) or pEB15 alone (Fig. 8, lane 1).

Transcriptional analyses. The complementation data had suggested that the *pilZ* promoter lay within ORF1, in the 770 bp between the *Kpn*I and *Sma*I restriction sites (Fig. 1 and 4).

FIG. 7. (A) Immunoblot of PilA located in whole-cell lysate samples of *P. aeruginosa* PAK (lane 1), R270[pUCP19] (lane 2), R270[pAB31] (lane 3), R270[pAB32] (lane 4), R270[pRIC355] (lane 5), and R270[pRIC364] (lane 6). (B) Surface-located fimbriae were extracted and immunoblotted with a 1:5,000 dilution of antifimbrial antiserum. The strains represented in each of the lanes are identical to those listed for panel A. The PilA protein is indicated on both immunoblots.

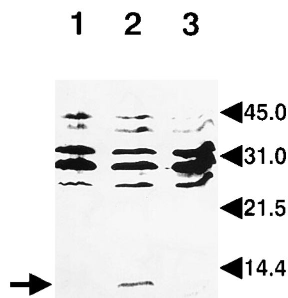

FIG. 8. Expression of the *pilZ* gene in *P. aeruginosa* ADD1976. Plasmid-encoded proteins produced from the T7 promoter in plasmid pEB15 were labelled with [^{35}S]methionine and separated on a 15% polyacrylamide gel. The plasmids used were pEB15 (lane 1), pRIC371 (lane 2), and pRIC372 (lane 3). The molecular mass markers are indicated, and the PilZ gene product produced by pRIC371 is indicated by an arrow.

Total RNA prepared from *P. aeruginosa* PAK[pUCP19] and PAK[pAB1] was hybridized in a Northern blot with the purified 465-bp insert from plasmid pRIC369 as a DNA probe (Fig. 9). No hybridizing bands could be detected from PAK-[pUCP19] (Fig. 9, lane 1), presumably because of the low level of mRNA transcript produced in a wild-type cell. However, when the *pilZ* gene was present on the multicopy plasmid pAB1, two hybridizing bands were detected (Fig. 9, lane 2), presumably because the level of specific mRNA produced was increased. These mRNA transcripts had sizes of 2 and 0.9 kb. The larger transcript would place the initiation point upstream of the *Kpn*I site and would include both *holB** and *pilZ*. The smaller transcript of 0.9 kb would be contained within the 1.77-kb *Kpn*I fragment, and transcription from this promoter is likely therefore to account for the ability of the *Kpn*I fragment to complement the R270 mutant in the absence of the *lac* promoter. Examination of the nucleotide sequence 900 bp upstream of the transcriptional terminator distal to *pilZ* revealed the presence of a putative promoter sequence (Fig. 4). Both the -35 and -10 regions displayed reasonable similarity (four of six) to the σ^{70} consensus promoter sequences of TT GACA and TATAAT, respectively. The spacing between these regions of 16 bp also lies well within the range for efficient promoter activity.

DISCUSSION

The biogenesis of type 4 fimbriae in *P. aeruginosa* appears to be a complex process that involves a large number of genes. To date, these genes have been clustered in three separate regions on the genome. In this report, we have identified a new gene whose product is crucial for fimbrial biogenesis and which is physically unlinked to any of the previously reported fimbrial genes. The 11.9-kDa fimbria-specific product of the *pilZ* gene represents a novel protein, since it has no significant homology with any existing database entry. It has been proposed that the infrastructural machinery for production and assembly of type 4 fimbriae is strongly conserved among the pathogens that produce this specific class of fimbriae. This is based on the evidence that the structural subunit from a variety of type 4 fimbriate bacteria can be produced and assembled in *P. aeruginosa* (3, 12, 19, 27). For this reason, we investigated the possibility that a *pilZ* homolog exists in other species that produce type 4 fimbriae. The homologs of other *pil* genes have been detected in several type 4 fimbriate species with the *Pseudomonas* genes as probes despite the high G+C content levels of *P. aeruginosa* (20, 24). However, when the *pilZ* gene was used to probe chromosomal digests of *N. gonorrhoeae*, *D. nodosus*, *Moraxella* spp., *Kingella* spp. and *Eikenella corrodens* under low stringency, no hybridizing bands could be detected (data not shown).

The precise role that PilZ plays in type 4 fimbrial biogenesis remains unclear. We have established that the R270 mutant is unaltered in its ability to produce a membrane pool of processed PilA monomers, indicating that the fimbrial biogenesis pathway is interrupted at the export-assembly step. On the basis of its predicted amino acid sequence and the corresponding Kyte-Doolittle hydropathy plot (22), PilZ is likely to be located cytoplasmically or in association with the inner membrane.

Transcription of *pilZ* occurs at low levels in the wild-type cell, which is indicated by the inability to detect a specific mRNA species unless additional copies are present on a multicopy plasmid (Fig. 9). With the exception of the structural subunit gene *pilA*, which is transcribed at high levels, detection of *pil*-specific mRNA species has been difficult because of the apparent low abundancy of these transcripts (2, 45, 47, 48). We have identified two *pilZ*-specific mRNA transcripts, one within the 1.77-kb *Kpn*I fragment, and a larger one that presumably contains *holB** and *pilZ* together. This would be consistent with the observation that the *holB** and *pilZ* sequences are separated by only 33 bp, with no obvious transcription termination or promoter signals within this interval. The smaller mRNA species is predominant, with the ratio of the larger-to-smaller mRNA transcripts being 1:3.8, as determined by densitometry. This suggests that the primary promoter for *pilZ* is the one identified at approximately 500 bp upstream of the initiation codon, within the *holB** coding sequence (Fig. 4). The idea that this is a functional promoter in vivo is supported by the fact that *pilZ* transcription from this promoter was sufficient to complement the R270 mutant back to wild type. Complementation of *pil* mutants back to wild-type levels of twitching motility is often not achieved (2), presumably because of subtle changes in expression levels caused by gene dosage effects on multicopy plasmids. This suggests that overexpression of PilZ is not detrimental to the biogenesis and function of the fimbrial filaments. Therefore, it seems unlikely that PilZ is a member of a multicomponent structure which requires the expression of its components in strict stoichiometric amounts (2, 30).

The identification of the *pilZ* locus on the 290-kb *Spe*I fragment I locates it on the same fragment as the *xcp* genes which are required for extracellular protein secretion, a system which bears multiple similarities to the type 4 fimbrial biogenesis system (2, 17, 26a). However, a *pilZ* mutant is unaltered in

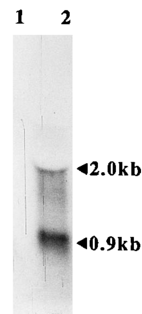

FIG. 9. Northern blot of *P. aeruginosa* RNA. Total RNA from *P. aeruginosa* PAK[pUCP19] (lane 1) and *P. aeruginosa* PAK[pAB1] (lane 2) was transferred to a nylon membrane and hybridized with a *pilZ*-specific probe. The two hybridizing transcripts are indicated, along with their respective sizes.

extracellular protein secretion. The *Spe*I fragment I is now the fourth region on the chromosome to contain *pil*-associated genes and from initial characterization does not lie adjacent to any other genes that are directly required for correct fimbrial biogenesis.

The identification of *pilZ* brings the total number of genes known to be involved in type 4 fimbrial biogenesis to 22. It is clear that this system is closely related to those involved in protein secretion and DNA uptake, as well as some aspects of phage biogenesis, but it also appears to have a number of unique components, including PilZ. The expression and function of fimbriae are regulated by a two-component sensor regulator system (PilS and PilR [16]) and a set of chemotaxis genes (PilGHIJK [8–10]).

There have been a number of other tantalizing chromosomal connections between type 4 fimbrial genes and those related to cell polarity, cell division, cell wall biosynthesis, and aromatic amino acid metabolism (1, 17, 26). The observation that *pilZ* is transcriptionally coupled to a gene encoding a protein which is likely to be involved in DNA replication is especially intriguing. This relationship may be simply accidental, but it is interesting to note that other genes related to cell division have been identified in or near other fimbrial operons. The *pilM* gene encodes a protein with some domain homology to the FtsA and MreB proteins of *E. coli*, which are involved in cell division and rod shape determination (26). Furthermore, *orfX*, which is located between *clpB* and *pilSR* (16), displays high similarity to *sfhB* (31), which is a suppressor of *ftsH*, which is essential for bacterial cell growth (4, 43). These relationships may not be significant or may merely reflect some catalytic similarities between these proteins. Alternatively, it may be that fimbrial biogenesis is in fact linked subtly to aspects of cell growth and division, as well as to the synthesis and remodelling of the cell wall. While the picture of fimbrial biogenesis remains a puzzle, the identification of its pieces will enable, firstly, an analysis of their role and, secondly, the integration of this information into a composite view of the mechanism of fimbrial biogenesis and function.

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