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Characterization of Type IV Pilus Genes in Plant Growth-Promoting *Pseudomonas putida* WCS358

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In a search for factors that could contribute to the ability of the plant growth-stimulating *Pseudomonas putida* WCS358 to colonize plant roots, the organism was analyzed for the presence of genes required for pilus biosynthesis. The *pilD* gene of *Pseudomonas aeruginosa*, which has also been designated *xcpA*, is involved in protein secretion and in the biogenesis of type IV pili. It encodes a peptidase that processes the precursors of the pilin subunits and of several components of the secretion apparatus. Prepilin processing activity could be demonstrated in *P. putida* WCS358, suggesting that this nonpathogenic strain may contain type IV pili as well. A DNA fragment containing the *pilD* (*xcpA*) gene of *P. putida* was cloned and found to complement a *pilD* (*xcpA*) mutation in *P. aeruginosa*. Nucleotide sequencing revealed, next to the *pilD* (*xcpA*) gene, the presence of two additional genes, *pilA* and *pilC*, that are highly homologous to genes involved in the biogenesis of type IV pili. The *pilA* gene encodes the pilin subunit, and *pilC* is an accessory gene, required for the assembly of the subunits into pili. In comparison with the *pil* gene cluster in *P. aeruginosa*, a gene homologous to *pilB* is lacking in the *P. putida* gene cluster. Pili were not detected on the cell surface of *P. putida* itself, not even when *pilA* was expressed from the *tac* promoter on a plasmid, indicating that not all the genes required for pilus biogenesis were expressed under the conditions tested. Expression of *pilA* of *P. putida* in *P. aeruginosa* resulted in the production of pili containing *P. putida* PilA subunits.

Many *Pseudomonas* strains derived from the rhizosphere have been studied because of their plant growth-promoting properties (20, 35). In the case of *Pseudomonas putida* WCS358, this property is based mainly on siderophore-mediated iron deprivation of plant-deleterious microorganisms (2, 11). However, efficient root colonization is also essential for plant growth stimulation. It is assumed that bacterial cell surface structures are involved in adhesion to and colonization of plant roots. A *P. putida* WCS358 mutant lacking the O-antigenic side chain of the lipopolysaccharide was not impaired in adhesion (13). Other cell surface structures, such as flagella (12) or surface-exposed domains of outer membrane proteins like porin F (9), have been suggested to be involved in root colonization. We are investigating the possibility that pili, which have never been reported to be present on *P. putida* cells until now, and/or the production of exoproteins may have a role in the colonization process.

An organism closely related to *P. putida*, the opportunistic pathogen *Pseudomonas aeruginosa*, produces type IV cell surface pili (fimbriae), which are involved in adhesion to mammalian epithelial cells (14, 51). In addition, these retractile pili are responsible for a primitive type of motion known as twitching motility, which enables bacterial translocation on solid surfaces (6, 50). The pili are composed of identical subunits, encoded by the *pilA* gene. The N-terminal segments of type IV pilins produced by different bacteria are highly conserved (45). The pilins are synthesized as precursors containing a positively charged leader peptide 6 or 7 amino acid residues long, followed by a highly hydrophobic domain of about 25 amino acid residues. After the leader peptide is cleaved off, the new N-terminal residue, in general phenylalanine but methionine in the case of *Vibrio cholerae* pili, is methylated (18, 29). In *P. aeruginosa*, the genes *pilB*, *pilC*, and *pilD*, which are required for the biogenesis of the pili, have been identified (40). These genes are located adjacent to the pilin structural gene *pilA* but are divergently transcribed (see Fig. 3). The *pilD* gene, which has also been designated *xcpA*, encodes the peptidase that cleaves off the leader peptide of prepilin (41). In *P. aeruginosa*, the prepilin peptidase is also required for the two-step protein secretion process (3) (for a review, see reference 48). In addition to PilD (XcpA), this secretion mechanism requires the products of at least 11 other genes, *xcpPQRSTUVWXYZ* (1, 4, 17). The N termini of the proteins encoded by *xcpT*, *-U*, *-V*, and *-W* are very similar to the N termini of the type IV prepilins. Indeed, it has been shown that at least one of the pilin-like Xcp proteins was processed by the PilD (XcpA) peptidase (4). Furthermore, it was found that the PilB and PilC proteins are homologous to XcpR and XcpS, respectively. This suggests that type IV pilus biogenesis and the assembly of the Xcp secretion apparatus are closely related processes.

Southern hybridization experiments have shown that the *pilD* (*xcpA*) gene, as well as other *xcp* genes, is also present in other gram-negative bacteria, including nonpathogenic *Pseudomonas* species derived from soil, such as *P. putida* and *Pseudomonas fluorescens* (8). In this report, we describe the isolation of the prepilin peptidase gene of *P. putida* WCS358. Furthermore, DNA sequence analysis revealed the presence of other pilus genes, including the type IV pilin structural gene, in this organism. Heterologous expression experiments showed the presence of a functional peptidase and the formation of pili composed of *P. putida* PilA subunits in *P. aeruginosa*.

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TABLE 1. Bacterial strains used in this study

Species and strain	Relevant characteristics	Source or reference
E. coli		
PC2494	*hsdR thi* Δ(*lac-proAB*) *supE* (F′ *proA*$^+$*B*$^+$ *lacI*^{q}ZΔM15 *traD36*)	Phabagen collection
1046	*met supE supF hsdS recA*	36
P. putida WCS358	Wild-type isolate, Nx^r	20
P. aeruginosa		
PAO222	*ilv-226 his-4 lysA12 met-28 trp-6 proA82*	21
KS904	PAO222 *xcpA1*	52
DB2	Multipiliated PAO1	45

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used are listed in Table 1. Strains were grown in L broth (49) unless otherwise stated. Other media used were tryptic soy broth (Difco), King's B medium (30), *Pseudomonas* isolation agar (Difco), and minimal medium (34). *Escherichia coli* and *P. aeruginosa* were grown at 37°C, and *P. putida* was grown at 28°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added when required at a concentration of 0.5 mM. Antibiotics were used in the following concentrations (in micrograms per milliliter): ampicillin, 100 (*E. coli*); tetracycline, 15 (*E. coli*), 40 (*P. putida*), and 200 (*P. aeruginosa*); kanamycin, 25 (*E. coli*), 40 (*P. putida*), and 300 (*P. aeruginosa*); streptomycin, 100 (*E. coli*), 150 (*P. putida*), and 1,000 (*P. aeruginosa*); and nalidixic acid, 25 (*P. putida*).

Plasmids and DNA manipulations. All plasmids used are listed in Table 2. Plasmid isolation, restriction endonuclease digestion, end filling with the Klenow fragment of *E. coli* DNA polymerase I, and ligation with T4 DNA ligase were performed by standard procedures (36). Plasmid pAG1 was constructed by cloning the 10-kb *Hin*dIII fragment, which contains pilus genes of *P. putida* WCS358, from pPX851 into pUC18. Expression vector pUR6500HE is a derivative of pMMB67HE (19) carrying a kanamycin resistance gene inserted in the *bla* gene. The *pilA* gene was cloned in pUR6500HE in two steps. First, the 0.9-kb *Stu*I fragment carrying *pilA* was cloned in the *Sma*I site of pEMBL18, resulting in pAG2, in which *pilA* is oriented in a direction opposite to that of the *lac* promoter. Subsequently, the *pilA* gene was cloned as a *Bam*HI-*Eco*RI fragment in pUR6500HE. In the resulting plasmid, pAG3, *pilA* is under control of the IPTG-inducible *tac* promoter. *E. coli* was transformed with plasmids as described elsewhere (36). Chromosomal DNA was isolated as described previously (37). Nylon membranes, Hybond-N for colony blotting and Hybond-N+ for Southern blotting, were used as recommended by the manufacturer (Amersham). DNA probes were labeled with digoxigenin by using a random-primed labeling kit (Boehringer Mannheim), and hybridizations were performed at 68°C as described by the manufacturer of the labeling kit. The final washings were done at 68°C in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate). Blots were developed by using a chemiluminescent detection kit as described by the manufacturer (Boehringer Mannheim).

Plasmid mobilization. Broad-host-range plasmids were transferred from *E. coli* 1046 to *Pseudomonas* strains by triparental mating by using helper plasmid pRK2013. *P. aeruginosa* and *P. putida* transconjugants were selected on *Pseudomonas* isolation agar and King's B medium, respectively, supplemented with the appropriate antibiotics.

DNA sequencing. DNA fragments were obtained from pAG1 and subcloned in pEMBL18 or -19 phagemids. Unidirectional deletions were made by using the double-stranded Nested Deletion Kit (Pharmacia). Single-stranded DNA was prepared from *E. coli* PC2494 carrying pEMBL derivatives after superinfection with helper phage VCSM13 (Stratagene). The nucleotide sequence of single-stranded DNA templates was determined by using the Gene-ATAQ Sequencing Kit (Pharmacia). The label used was [α-^{35}S]dATPαS (Amersham).

In vivo expression. Expression of the genes cloned under the control of the T7 promoter in plasmid pJX3 was analyzed in *P. putida* and *P. aeruginosa* by inducing the expression of T7 RNA polymerase encoded by pJRD7pol. Cells were grown in L broth at 30°C to an optical density at 660 nm (OD_{660}) of 0.8 (*P. aeruginosa*) or 1.5 (*P. putida*). Cells were washed and resuspended in minimal medium supplemented with 0.5% methionine assay medium (Difco) and 0.4% glucose and incubated for 1 h at 30°C. The synthesis of the T7 RNA polymerase was induced by shifting the temperature to 42°C. After 30 min, rifampin was added (final concentration, 200 μg/ml) to inhibit the host RNA polymerase, and incubation was continued for 10 min. Next, the cultures were returned to 30°C and incubated for an additional 30 min. Proteins were labeled by adding [^{35}S]methionine (5 μCi; Amersham) to 200 μl of cells for 5 min, after which the cells were harvested by centrifugation. Subcellular fractionation was performed as described elsewhere (4). Briefly, cells were broken by ultrasonic treatment, followed by centrifugation to pellet the membranes. The membranes were resuspended in 2% Sarkosyl to solubilize the

TABLE 2. Plasmids used in this study

Plasmid(s)	Relevant characteristics	Source or reference
pUC18	Ap^r, *ori* ColE1, *lacI* ϕ80d*lacZ*	53
pEMBL18 and pEMBL19	Ap^r, phagemid	10
pRK2013	Km^r, *ori* ColE1, Tra$^+$ Mob$^+$	16
pLAFR3	Tc^r, IncP	46
pJRD253	Sm^r, IncQ, T7ϕ10 promoter	7
pJRD7pol	Tc^r Km^r, T7pol behind λp_L promoter, λcI_{857}, IncP	7
pUR6500HE	Km^r, *tac* promoter, *lacI*^{q} IncQ	L. Frenken
pJX3	*xcpSTU* of *P. aeruginosa* in pJRD253	4
pUP2	*pilD* (*xcpA*) of *P. aeruginosa* in pUC19	M. Bally
pPX816 and pPX851	pLARF3 cosmid clones carrying *pil* genes of *P. putida* WCS358	This study
pAG1	*pil* genes of *P. putida* in pUC18	This study
pAG2	*pilA* of *P. putida* in pEMBL18	This study
pAG3	*pilA* of *P. putida* in pUR6500HE	This study

inner membranes and then centrifuged to pellet the outer membranes. The inner membrane proteins were precipitated by adding 1 volume of chloroform and 2.4 volumes of methanol.

SDS-PAGE. Protein samples were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl [pH 6.8], 0.02% bromophenol blue), heated at 95°C for 5 min, and separated by SDS-PAGE (33) on 15% acrylamide gels.

Enzyme assays and phage sensitivity test. Plate assays for elastase, lipase, and phospholipase C activity were performed as described previously (8). Sensitivity to the *P. aeruginosa* pilus-specific phage PO4 (5) was tested as described previously (40).

Pilus preparations. Cells, grown on L broth agar plates, were resuspended in 10 ml of 1× SSC to an OD_{660} of 2 to 4. Pili were sheared from the cells by passing the suspension three times through a 21-gauge needle (40). The cells were removed from the suspension by repeated centrifugation at 6,000 × *g* for 10 min until no pellet was visible. The pH of the supernatant was adjusted to 4.3 with 1 M citric acid (44). After precipitation at 4°C for 4 h, pili were pelleted at 6,000 × *g* for 15 min and resuspended in SDS-PAGE sample buffer.

Immunoblotting. Proteins were blotted from polyacrylamide gels onto nitrocellulose membranes (Schleicher & Schuell). The blots were blocked for 1 h in 0.5% nonfat dried milk (Protifar; Nutricia) in phosphate-buffered saline (PBS), pH 7.0, followed by incubation for 1 h with rabbit antiserum against PAO pilin in 0.1% Protifar in PBS. The blots were washed in both PBS and water for 10 min each. Subsequently, the blots were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G in 0.1% Protifar in PBS for 1 h and then washed as before. Detection was performed by using 4-chloro-1-naphthol and H_2O_2 as substrate for peroxidase (30 mg of 4-chloro-1-naphthol dissolved in 9 ml of methanol, with addition of 51 ml of PBS and 30 μl of 35% H_2O_2).

Electron microscopy. Cells were grown on L broth agar, tryptic soy broth agar, or King's B medium agar plates overnight and prepared for electron microscopy as described elsewhere (44), except that staining was performed with 1% uranyl acetate.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession number X74276.

RESULTS

Prepilin peptidase activity in *P. putida* WCS358. In *P. aeruginosa*, the PilD (XcpA) prepilin peptidase is required both for type IV pilus biogenesis and for secretion of most proteins (3). It cleaves off the short leader peptide of the pilin precursor and of pilin-like components of the protein secretion machinery. Southern hybridization experiments have shown that *P. putida* WCS358 contains *pilD* (*xcpA*)-related DNA (8). To analyze whether a functional prepilin peptidase is produced, an in vivo expression experiment was performed. Plasmid pJX3 contains the *xcpS*, *-T*, and *-U* genes of *P. aeruginosa*, cloned in vector pJRD253 under control of the inducible T7 promoter. After induction of the T7 promoter, only the pilin-like XcpT protein (16a) (and not XcpU as suggested in reference 4) is clearly detected. Bally et al. (4) have shown that this XcpT protein is processed in wild-type *P. aeruginosa* but not in a *pilD* (*xcpA*) mutant. To detect possible processing activity in *P. putida*, pJX3 was mobilized into strain WCS358 and, as a control, into *P. aeruginosa* PAO222 (Xcp^+) and KS904 (*xcpA1*). All strains also contained pJRD7pol encoding the T7 RNA polymerase. Strains were grown to an OD_{660} of 0.8 and pulse labeled as described in Materials and Methods. Both precursor and mature XcpT proteins could be detected in *P. putida* (Fig. 1A). However, only a small fraction of the total amount of XcpT protein was processed to the mature form under these conditions. Recently, it appeared that the expression of *xcp* genes in *P. aeruginosa* is induced at entry to the stationary phase of growth (1). Since this could also be the case for the *pilD* (*xcpA*) gene, the experiment was repeated, but this time *P. putida* cells were grown to an OD_{660} of 1.5. Under these conditions, processing was more efficient (Fig. 1B). Moreover, subcellular fractionation of the *P. putida* cells revealed that the precursor XcpT protein was present mainly in the fraction containing the cytoplasmic membrane proteins, whereas the mature product was located mainly in the outer membrane fraction (Fig. 1B). These different fractionation behaviors of precursor and mature XcpT were also found in the natural host, *P. aeruginosa* (4). The results indicate that *P. putida* produces a functional prepilin peptidase that is able to correctly process a heterologous prepilin-like protein.

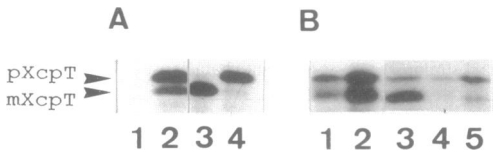


FIG. 1. Prepilin peptidase activity in *P. putida* WCS358. All strains contained pJRD7pol encoding the T7 RNA polymerase. pJX3 contains the *xcpT* gene of *P. aeruginosa*, cloned in vector pJRD253 under control of the T7 promoter. (A) Cells were grown to an OD_{660} of 0.8 and pulse labeled as described in Materials and Methods. Lane 1, *P. putida* WCS358(pJRD253); lane 2, *P. putida* WCS358(pJX3); lane 3, *P. aeruginosa* PAO222(pJX3); lane 4, *P. aeruginosa xcpA1* mutant KS904(pJX3). (B) *P. putida* WCS358(pJX3) was grown to an OD_{660} of 0.8 (lane 1) or 1.5 (lanes 2 through 5) before induction of T7 RNA polymerase. Lanes 1 and 2, total cells; lane 3, outer membrane fraction; lane 4, soluble fraction; lane 5, cytoplasmic membrane fraction. The positions of the precursor (pXcpT) and mature (mXcpT) forms of XcpT are indicated at left.

Cloning of the *pilD* (*xcpA*) gene of *P. putida* WCS358. To clone *pilD* (*xcpA*) and possibly additional flanking pilus genes of *P. putida*, a gene bank of strain WCS358 in pLAFR3 in *E. coli* was screened by colony hybridization. A 1.1-kb *Eco*RI-*Hin*dIII fragment from pUP2, carrying the *pilD* (*xcpA*) gene of *P. aeruginosa*, was used as a DNA probe. Approximately 1,000 colonies were screened, and five positive clones were identified. Plasmid DNA was prepared from these positive clones and further analyzed by restriction endonuclease digestion and Southern hybridization. All clones had an approximately 10-kb *Hin*dIII fragment in common which hybridized with the *pilD* (*xcpA*) probe. A similar fragment was present in *Hin*dIII-digested chromosomal DNA of *P. putida* (results not shown).

Complementation of a *pilD* (*xcpA*) mutation in *P. aeruginosa*. To analyze whether the isolated cosmid clones, carrying DNA of *P. putida* which hybridized with the *pilD* (*xcpA*) probe, encode a functional prepilin peptidase, complementation experiments were performed. The *P. aeruginosa xcpA1* mutant KS904 is not piliated and is also affected in the secretion of most proteins, including elastase, lipase, and phospholipase C. The cosmid clones were mobilized into this *xcpA1* mutant, and the transconjugants were assayed for protein secretion and piliation. Elastase plate assays indeed revealed that secretion was restored, as shown for clones pPX816 and pPX851 (Fig. 2).

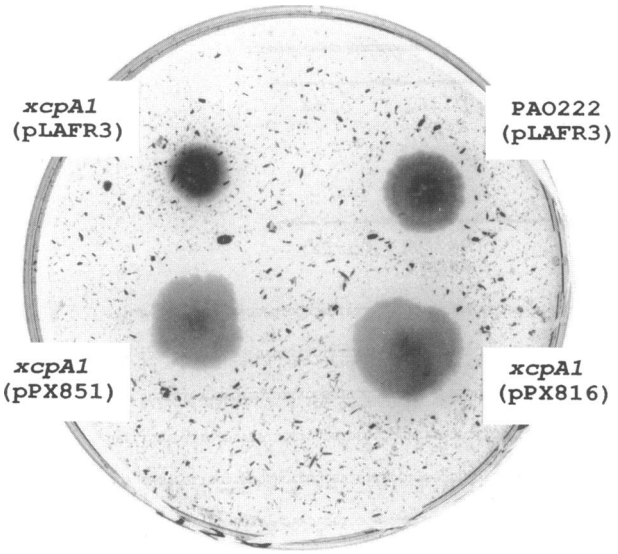


FIG. 2. Plate assay demonstrating elastase secretion. *P. aeruginosa* PAO222 (Xcp^+ Pil^+) and prepilin peptidase mutant KS904 (*xcpA1*) were mobilized with cloning vector pLAFR3 or with cosmid clone pPX816 or pPX851 carrying *pilD* (*xcpA*) of *P. putida* WCS358. A halo around the colonies shows the degradation of elastin in the plates. Also note the compact size of the colony of the *xcpA1* mutant carrying vector pLAFR3 compared with the sizes of the other colonies, which are more spread, probably because of twitching motility.

Similar results were found in plate assays for lipase and phospholipase C (results not shown). Figure 2 also indicates that piliation was restored. Because of the twitching motility caused by the pili, the colony of the wild-type *P. aeruginosa* is flat and spread on the surface of the plate, whereas the *xcpA1* mutant, which does not assemble pili, grows as a compact, dome-shaped colony. Introduction of the *pilD* (*xcpA*) gene of *P. putida* in the *xcpA1* mutant resulted in wild-type colony morphology (Fig. 2). The presence or absence of the retractile pili was also analyzed by using the *P. aeruginosa* pilus-specific phage PO4. The *xcpA1* mutant was resistant, whereas the wild type and the *xcpA1* mutant containing *pilD* (*xcpA*) of *P. putida* were sensitive (results not shown). Hence, the cloned *pilD* (*xcpA*) gene of *P. putida* encodes a functional prepilin peptidase and restores type IV pilus biogenesis and protein secretion in the *P. aeruginosa xcpA1* mutant.

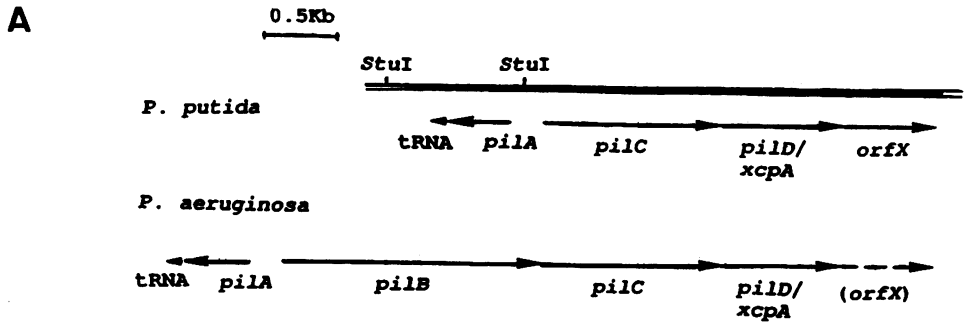
Nucleotide sequence analysis. The 10-kb *Hin*dIII fragment of pPX851, containing the *pilD* (*xcpA*) gene of *P. putida* WCS358, was subcloned in pUC18, resulting in pAG1. The location of the *pilD* (*xcpA*) gene was determined more accurately by restriction mapping and Southern hybridizations (results not shown). Subsequently, appropriate DNA fragments were cloned in pEMBL18 or -19 to determine the nucleotide sequence. The *pilD* (*xcpA*) gene was found to encode a protein of 288 amino acid residues (Fig. 3B), with 63.9% identity to the 290-amino-acid-residue-long PilD (XcpA) protein of *P. aeruginosa* (Fig. 4). The *pilD* (*xcpA*) gene of *P. aeruginosa* is preceded by two additional genes required for pilus biogenesis, i.e., *pilB* and *pilC* (Fig. 3A). Moreover, upstream of *pilB* in the opposite transcriptional orientation, the *pilA* gene encoding the pilin subunit is located (Fig. 3A). To identify possible homologs in *P. putida*, the region adjacent to *pilD* (*xcpA*) was analyzed. The nucleotide sequence of a DNA fragment of a total of 3.6 kb, determined on both strands, is shown in Fig. 3B and C. Immediately upstream of *pilD* (*xcpA*), a *pilC* homolog was identified (Fig. 3B). The gene encodes a protein 401 amino acid residues long that is homologous (43.1% identity) over its entire length with the 406-amino-acid-residue-long PilC protein of *P. aeruginosa* (Fig. 5).

Remarkably, the nucleotide sequence upstream of *pilC* did not reveal an open reading frame homologous to *pilB*. However, separated by an intergenic region of 218 bp, a pilin structural gene, *pilA*, was identified, oriented in the opposite direction to *pilC* and *pilD* (*xcpA*) (Fig. 3C). The 136-amino-acid-residue-long PilA protein has the characteristics of type IV prepilins; namely, a positively charged leader peptide 6 amino acid residues long followed by a stretch of hydrophobic residues which is highly conserved in type IV pilins of different gram-negative bacteria (Fig. 6). In *P. aeruginosa* and other species, it has been shown that the leader peptide is cleaved off after the glycine and that the new N-terminal phenylalanine is methylated (18, 45). In *P. putida*, the first amino acid after the cleavage site is isoleucine (Fig. 6). Furthermore, the 130-amino-acid-residue-long mature PilA contains the invariant glutamate at position 5 and two cysteine residues close to the C terminus, which possibly form a disulfide bridge (Fig. 6).

Downstream of *pilA*, a putative threonine tRNA gene including the promoter was found (Fig. 3C). The tRNA molecule is predicted to be 76 bases long and contains a CGU anticodon. A highly homologous $tRNA^{Thr}$ (CGU) gene has been identified downstream of *pilA* in *P. aeruginosa* (23). Downstream of the tRNA gene, an imperfect inverted repeat followed by four T's that possibly functions as a transcriptional terminator can be discerned (Fig. 3C).

It was noticed that the stop codon of *pilD* (*xcpA*) overlaps with a start codon of an open reading frame, designated *orfX* (Fig. 3B). The protein potentially encoded by *orfX* contains a putative ATP-binding site close to the N terminus. Comparison with the *P. aeruginosa* sequence downstream of *pilD* (*xcpA*) (reference 2a and our own unpublished results) revealed strong homology (results not shown). Whether the product of this putative gene is involved in pilus biogenesis and/or protein secretion in *P. putida* and *P. aeruginosa* requires further investigation. It should be noted, however, that the products of the *pilA*, *-B*, *-C*, and *-D* (*xcpA*) genes are probably not sufficient for pilus biogenesis, since additional genes have been shown to be required for the assembly of type IV pili in *V. cholerae* (42).

Heterologous expression of *P. putida pilA* in *P. aeruginosa*. The nucleotide sequence analysis revealed the presence of type IV pilus genes in *P. putida*. To detect pili, cells were examined by electron microscopy. However, after growth on plates of several standard laboratory media (L broth agar, tryptic soy broth agar, and King's B medium agar), pili were not detected on the cell surface. Similarly, introduction of plasmid pAG3, carrying the *pilA* gene of *P. putida* under control of the *tac* promoter, into strain WCS358 did not result in the detection of pili (results not shown). To identify the product encoded by the *P. putida pilA* gene and to determine whether it can assemble into pili, heterologous expression experiments with *P. aeruginosa* were performed. It has been shown that multipiliated *P. aeruginosa* strains are able to assemble type IV pili composed of pilin subunits from other gram-negative bacteria (24). Plasmid pAG3 was mobilized into the multipiliated strain DB2 and into the normally piliated strain PAO222. Transconjugants were grown on plates containing IPTG, and pili were isolated as described in Materials and Methods. Isolated pili were analyzed by SDS-PAGE and immunoblotting (Fig. 7). In the case of the strains containing the cloning vector, only the host PilA protein was detected (Fig. 7, lanes 1 and 3 in both panels). An additional protein, which was not recognized by the antiserum against PAO pilin (Fig. 7B, lane 2), was visible in the



B

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CATGAACGTTTTCCTTGTTTGATACGCATACACATTGGGGTCACTCTCGGCCCCAGGCTGGCAGCGGCGCAGTAGGCCTGAACAGACACCTTTTGTCACCCCTGCCCGCCTGGGCGTCAT 120
GTA ← pilA

CTTCGCTGAACTACTCTAGTGAGCATCAGGGAAGCACGCCCTCTCATGGACACGGCAAGCTGTTTCGAGGCTTGTCGTGCGGGCAAAAAAGGACCTTTGCATGAACCCTTCGATACGTCT 240
                                                                                                    pilC → M  N  F  S  I  R  L

TTACGCCTGGCAGGGCACCAACGCTGATGGCCTGGCCGTCAGTGGGCAAATGGCTGGGCGCAGCCCGGCTTATGTGCGCGCCGGGTTGCTGCGCCAGGGCATTCTGGTGGCGCGTTTCGG 360
  Y  A  W  Q  G  T  N  A  D  G  L  A  V  S  G  Q  M  A  G  R  S  F  A  I  V  R  A  G  L  L  R  Q  G  I  L  V  A  R  F  R

GCCTGCTGGGCGGGCGTGGCGTTGGCCGAAGCGGCGGGAGAAAACCGACCCGGCGGGTTTCAGCCGGCAGCTGGCGACTTTGCTGAAAGCCGGGGTGCCGCTGCTGCAGGCGTTCGAGGT 480
  F  A  G  R  A  W  R  W  P  K  R  R  E  K  T  D  P  A  G  F  S  R  Q  L  A  T  L  L  K  A  G  V  P  L  L  Q  A  F  E  V

GATGGGGCGCAGTGGCTGTGATGCGGCGCAGGCGGCGTTGCTGGCTCGATTGAAACAGGATGTGGCATCGGGGCTGGGCTGGGCTGGCGGATGCGTTGCAGCGCCACCCGGGGTGGTTCGATAC 600
  M  G  R  S  G  C  D  A  A  Q  A  A  L  L  A  R  L  K  Q  D  V  A  S  G  L  G  L  A  D  A  L  Q  R  H  F  G  W  F  D  T

GTTGTACTGCAACCTGGTGCGGGTGGGTGAGCAGTCCGGCACGCTCGACCGGCAGTTGGAGCAGTTGGCGGGCATGCTGGAACAGCGCTTGGCCCTGCACAAGAAGTTGCGCAAGGCGAT 720
  L  Y  C  N  L  V  R  V  G  E  Q  S  G  T  L  D  R  Q  L  E  Q  L  A  G  M  L  E  Q  R  L  A  L  H  K  K  L  R  K  A  M

GATCTACCCGCTGCTGTTGTTGCTGACCGGGTTGGGCGTTCGGCGGTTTGTTACTGGAGGTGATCCCGCAGTTTCAGAGCCTGTTCGCGGGGTTTGATGCGGCACTGCCGGCCTTTAC 840
  I  Y  P  L  L  L  L  L  T  G  L  G  V  S  A  V  L  L  E  V  I  P  Q  F  Q  S  L  F  A  G  F  D  A  A  L  P  A  F  T

CCAGTGGGTGATCGACTTGTCCACAGGGCTGGGGCGTCATGCACCGGTGTTGCTGGTAAATGCGGTGCTGTTGGCGGTGGCTGCGCGCGAGCTTTACCGCAAGCACAGGCCGGCGCGCTT 960
  Q  W  V  I  D  L  S  T  G  L  G  R  H  A  P  V  L  L  V  S  A  V  L  L  A  V  A  A  R  E  L  Y  R  K  H  R  P  A  R  L

GTGGATAACTCAGCGGGTGTTGGGCCTGCCGGTGTTCGGCAAGCTGCTGGGCAGGCGGCGCTGGCGCGTTTGCGCGCGCAGCCTGGCAACGTCTTATGCCGCTGGGGTGCCGCTGCTGGA 1080
  W  I  T  Q  R  V  L  G  L  P  V  F  G  K  L  L  G  Q  A  A  L  A  R  F  A  R  S  L  A  T  S  Y  A  A  G  V  P  L  L  D

TGCCTTGGGGACGGTGGCCAAGGCCAGTGGTGGCGAGCTGCATCAACAGGCGATCCAGCGCTTGCGCCAAGGCATGGCCAATGGGCAGGGGCTGAACCAGGCGATGGCGGCCGAACCGCT 1200
  A  L  G  T  V  A  K  A  S  G  G  E  L  H  Q  Q  A  I  Q  R  L  R  Q  G  M  A  N  G  Q  G  L  N  Q  A  M  A  A  E  P  L

GTTCCCGCCGCTGCTGGTGCAGCTGGTGGCCATCGGTGAGTCCAGTGGCACGCTGGACCAGATGCTGGAAAAAGCCGCCAGCCACTACGAGGAACAGGTCAGCCAGGCGCTGGACCAGTT 1320
  F  P  P  L  L  V  Q  L  V  A  I  G  E  S  S  G  T  L  D  Q  M  L  E  K  A  A  S  H  Y  E  E  Q  V  S  Q  A  L  D  Q  L

GACCAGCCTGCTGGAGCCGGCCATCGTGCTGGTCCTGGGCCTGCTGGTTGGCGGCCTGGTGGTGGCGATGTACCTGCCGATCTTCCAGCTGGGTAGCTTGATCTGAACATGACTTTATGG 1440
  T  S  L  L  E  P  A  I  V  L  V  L  G  L  L  V  G  G  L  V  V  A  M  Y  L  P  I  F  Q  L  G  S  L  I  -
                                                                                                    pilD/xcpA → M  T  L  W

ACCTTCCTGGCGATGGAGCCGGCTTACTTCATCACCCTGGCGACCGTGCTCGGCCTGTTGGTGGGCAGTTTCTCAACGTTGTGGTGTACCGCCTGCCGATCATGCTGGAGCGCCAATGG 1560
  T  F  L  A  M  E  P  A  Y  F  I  T  L  A  T  V  L  G  L  L  V  G  S  F  L  N  V  L  V  W  Y  R  L  P  I  M  L  E  R  Q  W

CAGCGTGAGGCACACGAAGTGCTGGGGCTGCCGGTGACCGAGCACGAGCGCTTCGACCTGTGCCTGCCGGCCTGCAGTCCACGCAATGCGGGCACCGGATACGGGCGTGGGAAAACCTC 1680
  Q  R  E  A  H  E  V  L  G  L  P  V  T  E  H  E  R  F  D  L  C  L  P  A  S  Q  C  T  Q  C  G  H  R  I  R  A  W  E  N  L

CCGGTGCTCAGCTACCTGGCGTTGCGCGGGCGCTGCTCGGCCTGCAAACAGCGCATCAGCGTGCGTTACCCGTTGGTCGAGGTGGGTTGTGCGCTGCTGTCGATGGTGGTGGCCTGGCGC 1800
  P  V  L  S  Y  L  A  L  R  G  R  C  S  A  C  K  Q  R  I  S  V  R  Y  P  L  V  E  V  G  C  A  L  L  S  M  V  V  A  W  R

TACGGGGCCAGCGTCGAGGCACTGGTGCTGCTGCCGCTGATCCTGGAGCTTGCTGGCTCTGAGCTTGATCGACCATGACCAACAACTGCTGCCCGAATGCCATCGTGCTGCCGGGGCTGTGG 1920
  Y  G  A  S  V  E  A  L  V  L  L  P  L  T  M  S  L  L  A  L  S  L  I  D  H  D  Q  Q  L  L  P  D  A  I  V  L  P  G  L  W

CTTGGGCTGATCGTCAATTACTTCGGGGTATGGGTGCCCTTGCCCGATGCCGTATGCGGCGCGGTGGTCGGGTACCTCAGCCTGTGGACGGTGTACTGGTTGTTCAAGCTGGTTACCGGC 2040
  L  G  L  I  V  N  Y  F  G  V  W  V  P  L  P  D  A  V  C  G  A  V  V  G  Y  L  S  L  W  T  V  Y  W  L  F  K  L  V  T  G

AAGGAAGGCATGGGCTATGGCGACTTCAAGCTGTTGGCGCTGCTCGGCGCCTGGGGTGGCTGGCAGGTGCTGCCGCTGACCTTGTTGCTGTCGTCGGTGCTTGGGGCGCTGGTTGGGGTG 2160
  K  E  G  M  G  Y  G  D  F  K  L  L  A  L  L  G  A  W  G  G  W  Q  V  L  P  L  T  L  L  L  S  S  V  L  G  A  L  V  G  V

TACCTGCTGCGGGTGCGCAACGATTCCATGGGTACGGCCATGCCATTTGGGCCCTATCTGGCAATTGCGGGGTGGATTGCCGTGCTCTGGGGTGATGAAATATACGCCTCTAACATGCAA 2280
  Y  L  L  R  V  R  N  D  S  M  G  T  A  M  P  F  G  P  Y  L  A  I  A  G  W  I  A  V  L  W  G  D  E  I  Y  A  S  N  M  Q

CTGCTTGGATTCTGATGACCACTGCAGCTTTCACCCCTTGGATTCTGGGCCTGACCGGCGAATCGGCAGCGGCAAGAGTGCCGCCGCCGAGCGCTTCGTCGAGCTTGGCGTGCACCTGG 2400
  L  L  G  F  -
        orfX → M  T  T  A  A  F  T  P  W  I  L  G  L  T  G  G  I  G  S  G  K  S  A  A  A  E  R  F  V  E  L  G  V  H  L

TCGATGCCGACCAGGCGGCGCGCTGGGTGGTCGAGCCTGGGCGACCGGCGCTGGCCAGCATTGTCGAGCGCTTCGGCCCTGGCGTGCTGCTGGACGATGGCCAACTCGACCGCGCGGCCT 2520
  V  D  A  D  Q  A  A  R  W  V  V  E  P  G  R  P  A  L  A  S  I  V  E  R  F  G  P  G  V  L  L  D  D  G  Q  L  D  R  A  A

TGCGCCAGCTGATTTTCGCCGACCCGGCGCAGCGCCAGTGGCTGGAGGCGTTGCTGCACCCGCTGATCGGGCAGGAGATTTTCAGCTACCTGGCCAAGGCCGAATCGCCTTATGCCGTGT 2640
  L  R  Q  L  I  F  A  D  P  A  Q  R  Q  W  L  E  A  L  L  H  P  L  I  G  Q  E  I  F  S  Y  L  A  K  A  E  S  P  Y  A  V

ATGTGTCACCCTTGCTGATCGAGTCTGGCCAGTATCGCAAGACCCAGCGCGTGCTGGTGATCGATGCGCCGCAGGCGTTGCAGATGGAACGTACGCTGCAGCGGGACAACACCAGCCCGG 2760
  Y  V  S  P  L  L  I  E  S  G  Q  Y  R  K  T  Q  R  V  L  V  I  D  A  P  Q  A  L  Q  M  E  R  T  L  Q  R  D  N  T  S  P

AGCAGGTGCAGGCCATTCTCAATGCGCAGCTGGCACGTGAAGAGCGGCTGCGCCATGCCGACGATGTGGTGGTCAATGACCGCGACCTTGCCGCGTTGCACGAGCAGATCGACCGTCTGC 2880
  E  Q  V  Q  A  I  L  N  A  Q  L  A  R  E  E  R  L  R  H  A  D  D  V  V  V  N  D  R  D  L  A  A  L  H  E  Q  I  D  R  L

ATCATTTTTACCTGACTTTACGAGGAGGCCAGCCATGAGCCAGCCATTGACCGTCGACTG 2940
  H  H  F  Y  L  T  L  R  G  G  Q  P  -
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C

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CATGCAAAGGTCCTTTTTTGCCCGCACGACAAGCCTCGAAACAGCTTGCCGTGTCCATGAGAGGGCGTGCTTCCCTGATGCTCACTAGAGTAGTTCAGCGAAGATGACGCCCAGGCGGGC 120
GTA ← pilC

                                    StuI
AGGGGTGACAAAAGGTGTCTGTTCCTGGCCTACTGCGCCGCTGCCAGCCTGGGGCCGAGAGTGACCCCAATGTGTATGCGTATCAAACAAGGAAAACGTTCATGAAAGGCAACGCGGTAT 240
                                                                                                    pilA → M  K  G  Q  R  G  I

CACCCTGATCGAACTGATGATCGTTGTCGCGATCATCGGCATTCTGGCGACCATCGCCATACCGATGTACACCAACCACCAGTCCCGCACCAAGGCCGCAGCCGGGTTGCTGGAGATTTC 360
  T  L  I  E  L  M  I  V  V  A  I  I  G  I  L  A  T  I  A  I  P  M  Y  T  N  H  Q  S  R  T  K  A  A  A  G  L  L  E  I  S

AGCGCTGAAGACAGCCATGGACCTGCGCTTGAATGAAGGCAAGGATGTGGCGGATGTAGGCGCGCTGGGTGGGCAACCGGCGACAGCGCATTGCGCGATTACCGCCAGCGGCAACGCAGC 480
  A  L  K  T  A  M  D  L  R  L  N  E  G  K  D  V  A  D  V  G  A  L  G  G  Q  P  A  T  A  H  C  A  I  T  A  S  G  N  A  A

TGCCGGCACCGGGAGCATTGTGTGCACACTGGTGGATGCACCGGCCACCGTGGTTGGCAAAGCCCTGACCCTGACCCGCTCGGCTACTGGGTGGGGCTGCACCACCAACATCGAGGAAGA 600
  A  G  T  G  S  I  V  C  T  L  V  D  A  P  A  T  V  V  G  K  A  L  T  L  T  R  S  A  T  G  W  G  C  T  T  N  I  E  E  D

CCTCGCGCCGAGTGGTTGCAAAGGGGCTTGAGGGCAGGGCAATAAACGAGGGGAAACAGGGGTTTGCGTAACGGGTGCGGCAGTGGTACGTTGCGCTACACGCCGGTGTACTCAGTCGG 720
  L  A  F  S  G  C  K  G  A  -                                                                     tRNA →

TAGAGCAGCGCACTCGTAACGCGAAGGTCGCAGGTCGATCCTGTCTCCGGCACCAGAGACCAGTTTCCAGAAGTCTATGACTGTCTACTAAACACCCAGAAAAGCCCGCCTAGTGCGG 840

GCTTTGCTGTTTCTGTCTGTCTCGTTTTGTCTCTGGGCATCTACGCGATTTAGGGATATGGTTAGGGATATC 912
```

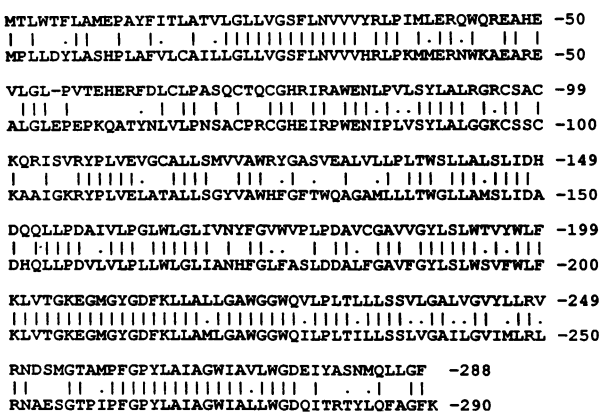

FIG. 4. Alignment of the PilD (XcpA) proteins of *P. putida* (top) and *P. aeruginosa* (bottom). Identical (|) and similar (·) amino acid residues are indicated. Similar amino acids are defined as being within the following groups: S and T; R and K; F, W, and Y; I, L, M, and V; D and E; and N and Q.

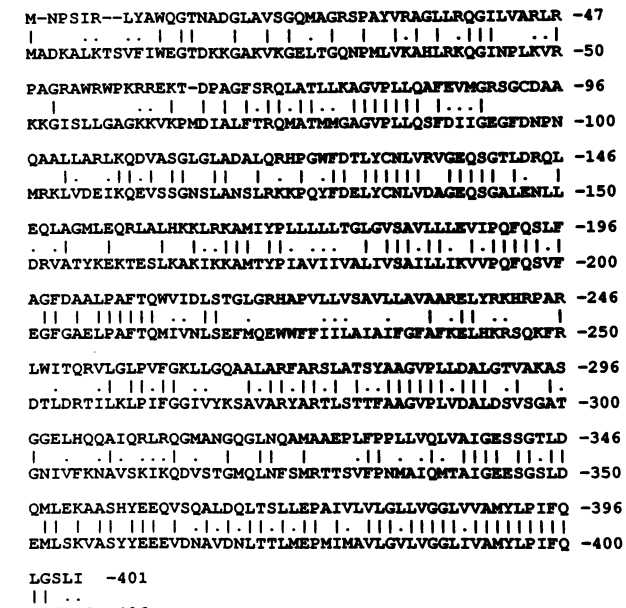

FIG. 5. Alignment of the PilC proteins of *P. putida* (top) and *P. aeruginosa* (bottom). Identical and similar residues are indicated as in Fig. 4.

case of strain DB2 containing pAG3 (Fig. 7A, lane 2). This additional protein, which most likely represents PilA of *P. putida*, migrated faster than the host PilA protein. This is consistent with the sequencing results, since the mature PilA of *P. putida* is predicted to be 130 amino acid residues long, whereas PilA of *P. aeruginosa* PAO is composed of 143 residues. When strain PAO222 was used, no *P. putida* PilA protein was detected and the biogenesis of host pili seemed to be inhibited to a large extent by the presence of the *P. putida* *pilA* gene (Fig. 7A, lane 4).

DISCUSSION

The potato root-isolated *P. putida* WCS358 has been characterized as a plant growth-promoting bacterium (2, 20). This phenomenon requires efficient root colonization, the molecular basis of which is still unclear. Bacterial cell surface structures such as pili, as well as secreted proteins, may have a role in this process. Hybridization experiments have shown that *P. putida* contains DNA related to the *pilD* (*xcpA*) gene of *P. aeruginosa* (8). The *pilD* (*xcpA*) gene encodes a prepilin peptidase that is required for the assembly of type IV pili and for protein secretion (3, 40, 41). Here, we characterized the *pilD* (*xcpA*) gene of *P. putida* WCS358, which was found to encode a functional product. Furthermore, upstream of *pilD* (*xcpA*), two additional genes connected with type IV pilus production appeared to be present; namely, *pilC* and *pilA*. The *pilC* gene encodes a hydrophobic protein and is probably located in the cytoplasmic membrane. The *pilA* gene encodes the pilin subunit and could be heterologously expressed and assembled into pili in *P. aeruginosa* (Fig. 7). The PilA protein has the characteristics of a type IV pilin subunit: it contains a positively charged leader peptide 6 amino acid residues long, followed by a stretch of hydrophobic amino acids, that is highly conserved among type IV pilins of different gram-negative bacteria (28, 45). The first amino acid after the predicted cleavage site is isoleucine. In most gram-negative bacteria producing type IV pili, the first residue of the mature pilin subunit is a phenylalanine, which becomes methylated after processing. However, the first residue of the mature pilin in *V. cholerae* is a methylated methionine (29). Moreover, substitution of the N-terminal phenylalanine in pilin of *P. aeruginosa* by other amino acids did not affect cleavage and assembly into pili, indicating that an N-terminal phenylalanine is not absolutely required (47). The mature pilin of *P. putida* contains a glutamate residue at position 5, which has been shown to be required for methylation and pilin assembly in *P. aeruginosa* (43, 47). Like other type IV pili, but unlike the pilin-like components of the secretion machinery, the C terminus of PilA contains two cysteine residues (residues 120 and 133 in precursor PilA) (Fig. 6) that probably form a disulfide bridge. It has been shown that the C terminus of *P. aeruginosa* PilA contains an epithelial cell-binding domain, a function that is enhanced by the formation of an intrachain disulfide bond (25). Similarly, the C terminus of the *P. putida* pilin might contain a domain involved in adhesion. The *P. putida* pilin contains two additional cysteine residues, located toward the central part of the protein (residues 78 and 95 in precursor PilA) (Fig. 6). As far as we know, this is the first time that type

FIG. 3. Nucleotide sequence and genetic organization of the identified pilus genes of *P. putida* WCS358. (A) Comparison of the pilus gene clusters of *P. putida* and *P. aeruginosa*. The nucleotide sequence downstream of *pilD* (*xcpA*) in *P. aeruginosa* has been only partly determined. The nucleotide sequence of the 3.6-kb DNA fragment of *P. putida*, indicated by the shaded bar, is shown in panels B and C. The indicated *Stu*I sites were used for subcloning of *pilA* under control of the *tac* promoter. (B) Sequence of *pilC*, *pilD* (*xcpA*), and *orfX*. The 221-bp sequence upstream of *pilC*, up to the start codon of the oppositely oriented *pilA* gene, is also shown. The putative ATP-binding site in OrfX is shown boxed. (C) Sequence of *pilA* and a putative $tRNA^{Thr}$ (CGU) gene. The 221-bp sequence upstream of *pilA*, which is complementary to the sequence upstream of *pilC* depicted in panel B, is also shown. The 76-bp $tRNA^{Thr}$ (CGU) gene, indicated in boldface type, starts at nucleotide 702 and ends at nucleotide 777. Underlined nucleotides upstream of the tRNA gene indicate the tRNA promoter (23). Horizontal arrows indicate an inverted repeat.

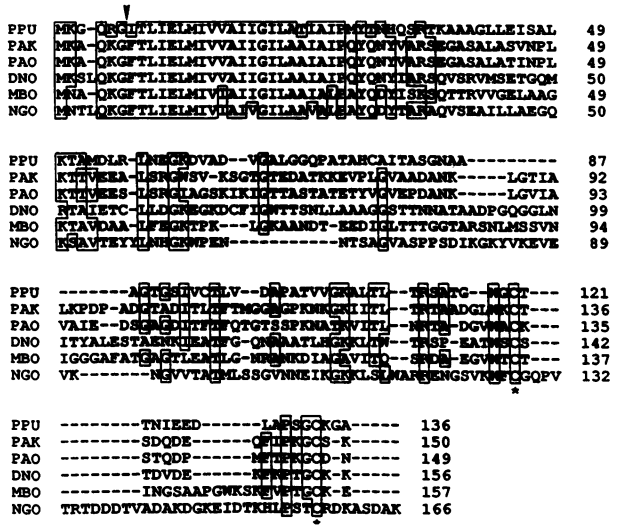

FIG. 6. Alignment of type IV prepilins of *P. putida* (PPU), *P. aeruginosa* PAK and PAO (45), *Dichelobacter nodosus* (DNO) (15), *Moraxella bovis* (MBO) (38), and *N. gonorrhoeae* (NGO) (39). Residues identical in at least four proteins are shown boxed. The two cysteine residues that (probably) form a disulfide bond are indicated by asterisks. The prepilin peptidase cleavage site is indicated by an arrowhead.

IV pilus genes have been detected in a nonpathogenic bacterium.

The *P. putida* PilA protein was found to be assembled into pili when expressed in the multipiliated *P. aeruginosa* DB2 but not when expressed in the normally piliated strain PAO222 (Fig. 7). Similar results have been described by Pasloske et al. (44), who found that a mutant pilin of *P. aeruginosa* PAK was assembled in the *P. aeruginosa* PAO-derived, multipiliated strain DB2 but not in a normally piliated PAO strain. These observations might be explained by the fact that the type IV pili are retractile by disassembly of the pili. In the normally piliated strains, disassembly results in a flow of pilin subunits back into a pool of subunits within the cell envelope. If assembly of host pilin subunits is favored over assembly of foreign pilins, pili composed of only host pilin subunits would be assembled. In the multipiliated strains, the pili are not disassembled and the host pilin subunits remain in the pilus structures. Consequently, a relatively high concentration of heterologous subunits will accumulate in the pool of subunits in the cell envelope, thus favoring their assembly into the pilus structures.

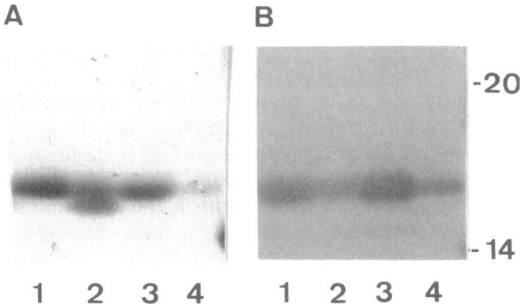

FIG. 7. Expression of *pilA* of *P. putida* in *P. aeruginosa*. *P. putida pilA* was cloned behind the *tac* promoter in pUR6500HE, resulting in pAG3. An SDS-PAGE gel (A) and an immunoblot (B) of purified pili are shown. For the immunoblot, an antiserum directed against *P. aeruginosa* pili was used. Lanes: 1, strain DB2(pUR6500HE); 2, DB2(pAG3); 3, PAO222(pUR6500HE); 4, PAO222(pAG3). At right, the positions of two molecular mass standard proteins (in kilodaltons) are indicated.

Although *P. putida* possesses type IV pilus genes, electron microscopy studies did not reveal the presence of pili on the cell surface. This suggests that the expression of the *pil* genes is strongly regulated. The demonstration that the prepilin-like XcpT protein of *P. aeruginosa* is processed when expressed in *P. putida* indicates that the *pilD* (*xcpA*) gene and probably also *pilC* and putative additional genes in the same operon are expressed. However, when *pilA* was expressed in *P. putida* from an inducible promoter on a plasmid, pili were still not detected, indicating that additional genes required for pilus biogenesis are absent or not expressed. One of these genes might be *pilB*, which is not present upstream of *pilC* in the *P. putida* gene cluster, in contrast to the genetic organization in *P. aeruginosa* (Fig. 3). However, it seems unlikely that *P. putida* WCS358 has lost the *pilB* gene but maintained other *pil* genes, including *pilA* and *pilC*, in its genome. Therefore, we favor the hypothesis that a *pilB* gene is present in *P. putida* but at a different chromosomal position. Alternatively, since *pilB* and *xcpR* are highly homologous, the XcpR homolog of *P. putida* might have a dual role and might, like PilD (XcpA), be involved both in pilus biogenesis and in protein secretion. The first hypothesis is supported by the cloning of two different DNA fragments by hybridization with a probe carrying the *xcpR* gene of *P. aeruginosa* (unpublished results). Preliminary DNA sequencing results showed that one clone contained *xcpR* and other *xcp* genes whereas the second clone may contain *pilB* (unpublished results). It should be noted that Koga et al. (31) recently reported that the *pilB* gene of *P. aeruginosa*, although located directly upstream of *pilC* and *pilD* (*xcpA*), does not form a transcriptional unit with these genes. Furthermore, it was recently reported that in *Neisseria gonorrhoeae* also, a *pilB* homolog is not arranged in tandem with *pilD* (*xcpA*)- and *pilC*-related genes (32).

In *P. aeruginosa*, transcription of *pilA*, but not of *pilB*, *-C*, and *-D* (*xcpA*), requires the sigma 54 RNA polymerase initiation factor RpoN (26, 31). The transcription is controlled by PilS and PilR, belonging to the family of two-component transcriptional regulatory systems (22, 27). The *P. putida* DNA sequence upstream of *pilA* does not show homology to promoters that are transcribed by RpoN-containing RNA polymerase (GG-N_{10}-GC), suggesting that expression is regulated differently in this bacterium. The results of the experiment demonstrating processing of XcpT (Fig. 1) suggest that the expression of *pilD* (*xcpA*) is growth phase dependent, since more processing was detected in the late logarithmic phase or at the beginning of stationary phase. Similarly, growth phase-dependent expression was detected for the *xcp* genes in *P. aeruginosa* (1). Another interesting possibility is that the expression of *pil* genes in *P. putida* is induced by compounds present in plant root exudate. The use of transcriptional fusions with reporter genes may be helpful in finding the conditions that induce pilus synthesis. The pili and the exoproteins of *P. putida* may have a role in adhesion to and colonization of the plant roots and therefore may contribute to the plant growth stimulation. This possibility will be investigated by inactivating the *pil* genes on the chromosome and studying the effect of these mutations on colonization and plant growth promotion.

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REFERENCES

1. **Akrim, M., M. Bally, G. Ball, J. Tommassen, H. Teerink, A. Filloux, and A. Lazdunski.** 1993. Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. **10**:431–443.
2. **Bakker, P. A. H. M., J. G. Lamers, A. W. Bakker, J. D. Marugg, P. J. Weisbeek, and B. Schippers.** 1986. The role of siderophores in potato tuber yield increase by *Pseudomonas putida* in a short rotation of potato. Neth. J. Plant Pathol. **92**:249–256.

2a. **Bally, M.** Personal communication.

3. **Bally, M., G. Ball, A. Badere, and A. Lazdunski.** 1991. Protein secretion in *Pseudomonas aeruginosa*: the *xcpA* gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. J. Bacteriol. **173**:479–486.
4. **Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen.** 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6**:1121–1131.
5. **Bradley, D. E.** 1973. A pilus dependent *Pseudomonas aeruginosa* bacteriophage with a long noncontractile tail. Virology **51**:489–492.
6. **Bradley, D. E.** 1980. A function of *Pseudomonas aeruginosa* PAO pili: twitching motility. Can. J. Microbiol. **26**:146–154.
7. **Davison, J., N. Chevalier, and F. Brunel.** 1989. Bacteriophage T7 RNA polymerase-controlled specific gene expression in *Pseudomonas*. Gene **83**:371–375.
8. **De Groot, A., A. Filloux, and J. Tommassen.** 1991. Conservation of *xcp* genes, involved in the two-step protein secretion process, in different *Pseudomonas* species and other gram-negative bacteria. Mol. Gen. Genet. **229**:278–284.
9. **De Mot, R., P. Proost, J. Van Damme, and J. Vanderleyden.** 1992. Homology of the root adhesin of *Pseudomonas fluorescens* OE 28.3 with porin F of *P. aeruginosa* and *P. syringae*. Mol. Gen. Genet. **231**:489–493.
10. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. **11**:1645–1655.
11. **De Weger, L. A., J. C. H. M. van Arendonk, K. Recourt, G. A. J. M. van der Hofstad, P. J. Weisbeek, and B. Lugtenberg.** 1988. Siderophore-mediated uptake of Fe^{3+} by the plant growth-stimulating *Pseudomonas putida* strain WCS358 and by other rhizosphere microorganisms. J. Bacteriol. **170**:4693–4698.
12. **De Weger, L. A., C. I. M. van der Vlugt, A. H. M. Wijffjes, P. A. H. M. Bakker, B. Schippers, and B. Lugtenberg.** 1987. Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. J. Bacteriol. **169**:2769–2773.
13. **De Weger, L. A., M. C. M. van Loosdrecht, H. E. Klaassen, and B. Lugtenberg.** 1989. Mutational changes in physicochemical cell surface properties of plant growth-stimulating *Pseudomonas* spp. do not influence the attachment properties of the cells. J. Bacteriol. **171**:2756–2761.
14. **Doig, P., T. Todd, P. A. Sastry, K. K. Lee, R. S. Hodges, W. Paranchych, and R. T. Irvin.** 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect. Immun. **56**:1641–1646.
15. **Elleman, T. C., P. A. Hoyne, N. M. McKern, and D. J. Stewart.** 1986. Nucleotide sequence of the two-subunit pilin of *Bacteroides nodosus* 265. J. Bacteriol. **167**:243–250.
16. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76**:1648–1652.

16a. **Filloux, A.** Unpublished data.

17. **Filloux, A., M. Bally, G. Ball, M. Akrim, J. Tommassen, and A. Lazdunski.** 1990. Protein secretion in Gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. EMBO J. **9**:4323–4329.
18. **Frost, L. S., M. Carpenter, and W. Paranchych.** 1978. N-methylphenylalanine at the N-terminus of *Pseudomonas aeruginosa* K pili. Nature (London) **271**:87–89.
19. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48**:119–131.
20. **Geels, F. P., and B. Schippers.** 1983. Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatment with antagonistic fluorescent *Pseudomonas* spp. Phytopathol. Z. **108**:207–214.
21. **Haas, D., and B. W. Holloway.** 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144**:243–251.
22. **Hobbs, M., E. S. R. Collie, P. D. Free, S. P. Livingston, and J. S. Mattick.** 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. **7**:669–682.
23. **Hobbs, M., B. Dalrymple, S. F. Delaney, and J. S. Mattick.** 1988. Transcription of the fimbrial subunit gene and an associated transfer RNA gene of *Pseudomonas aeruginosa*. Gene **62**:219–227.
24. **Hoyne, P. A., R. Haas, T. F. Meyer, J. K. Davies, and T. C. Elleman.** 1992. Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. J. Bacteriol. **174**:7321–7327.
25. **Irvin, R. T., P. Doig, K. K. Lee, P. A. Sastry, W. Paranchych, T. Todd, and R. S. Hodges.** 1989. Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. Infect. Immun. **57**:3720–3726.
26. **Ishimoto, K. S., and S. Lory.** 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA **86**:1954–1957.
27. **Ishimoto, K. S., and S. Lory.** 1992. Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. J. Bacteriol. **174**:3514–3521.
28. **Johnson, K., M. L. Parker, and S. Lory.** 1986. Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. J. Biol. Chem. **261**:15703–15708.
29. **Kaufman, M. R., J. M. Seyer, and R. K. Taylor.** 1991. Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. Genes Dev. **5**:1834–1846.
30. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
31. **Koga, T., K. Ishimoto, and S. Lory.** 1993. Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. Infect. Immun. **61**:1371–1377.
32. **Lauer, P., N. H. Albertson, and M. Koomey.** 1993. Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in *Neisseria gonorrhoeae*. Mol. Microbiol. **8**:357–368.
33. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
34. **Lugtenberg, B., R. Peters, H. Bernheimer, and W. Berendsen.** 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. **147**:251–262.
35. **Lugtenberg, B. J. J., and L. A. de Weger.** 1992. Plant root colonization by *Pseudomonas* spp., p. 13–19. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*. American Society for Microbiology, Washington, D.C.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.
37. **Marmur, J.** 1961. A procedure for the isolation of deoxynucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
38. **Marrs, C. F., G. Schoolnik, M. Koomey, J. Hardy, J. Rothbard, and S. Falkow.** 1985. Cloning and sequencing of a *Moraxella bovis* pilin gene. J. Bacteriol. **163**:132–139.
39. **Meyer, T. F., E. Billyard, R. Hass, S. Storzbach, and M. So.** 1984. Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA **81**:6110–6114.
40. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172**:2911–2919.
41. **Nunn, D. N., and S. Lory.** 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA **88**:3281–3285.
42. **Ogierman, M. A., S. Zabihi, L. Mourtzios, and P. A. Manning.** 1993. Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. Gene **126**:51–60.
43. **Pasloske, B. L., and W. Paranchych.** 1988. The expression of mutant pilins in *Pseudomonas aeruginosa*: fifth position glutamate affects pilin methylation. Mol. Microbiol. **2**:489–495.
44. **Pasloske, B. L., D. G. Scraba, and W. Paranchych.** 1989. Assembly of mutant pilins in *Pseudomonas aeruginosa*: formation of pili composed of heterologous subunits. J. Bacteriol. **171**:2142–2147.
45. **Sastry, P. A., B. B. Finlay, B. L. Pasloske, W. Paranchych, J. R. Pearlstone, and L. B. Smillie.** 1985. Comparative studies of the amino acid and nucleotide sequences of pilin derived from *Pseudomonas aeruginosa* PAK and PAO. J. Bacteriol. **164**:571–577.
46. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169**:5789–5794.
47. **Strom, M. S., and S. Lory.** 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*. Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. J. Biol. Chem. **266**:1656–1664.
48. **Tommassen, J., A. Filloux, M. Bally, M. Murgier, and A. Lazdunski.** 1992. Protein secretion in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **103**:73–90.
49. **Tommassen, J., H. van Tol, and B. Lugtenberg.** 1983. The ultimate localization of an outer membrane protein of *Escherichia coli* K-12 is not determined by the signal sequence. EMBO J. **2**:1275–1279.
50. **Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick.** 1990. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101**:33–44.
51. **Woods, D. E., D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29**:1146–1151.
52. **Wretlind, B., and O. R. Pavlovskis.** 1984. Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. J. Bacteriol. **158**:801–808.
53. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.