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 pili 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 pili 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 reticulate 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 phenyllalnine 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, xcpPQRSTUWXYZ (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 pilins. 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 pili 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.
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' lacFZΔM15 traD36) | Phabagen collection |
| 1046               | met supE supF hsdS recA 36 |                     |
| P. putida WCS358   | Wild-type isolate, Nv' | 20                  |
| P. aeruginosa PAO222 | ilv-226 his-4 lysA12 met-28 trp-6 proA12        | 21                  |
| KS904             | PAO222 xcpAI             | 52                  |
| DB2                  | Multiplicated 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 aeruginosa 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 HindIII 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 StuI fragment carrying pilA was cloned in the Smal 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 BamHI-EcoRI 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 plasmidics. 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-35S]dATP (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 (OD660) 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 [35S]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', ori ColE1, lacI 480delZ | 53                  |
| pEMBL18 and pEMBL19| Ap', phagemid             | 10                  |
| pRK2013            | Km', ori ColE1, Tra+ Mob+  | 16                  |
| pLAFR3             | Tc', IncP                 | 46                  |
| pJRD253            | Sm', IncQ, T7610 promoter | 7                   |
| pJRD7pol           | Tc' Km', T7pol behind xps, promoter, xcl57, IncP | 7                   |
| pUR6500HE          | Km', tac promoter, lacT+ IncQ | L. Frenken        |
| pJX3               | xcpSTU of P. aeruginosa in pRJ253 | 4                   |
| pUP2               | pilD (xcpA) of P. aeruginosa in pUC19 | M. Bally        |
| pPX816 and pPX851  | pLAFR3 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% bromphenol 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_{600} of 2 to 4. Pili were sheared from the cells by passing the suspension three times through 21-gauge needles (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_{2}O_{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_{2}O_{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 P1D (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 pJR253 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 (XcpA+) and KS904 (xcpA1). All strains also contained plJRD7pol encoding the T7 RNA polymerase. pJX3 contains the xcpT gene of *P. aeruginosa*, cloned in vector pJR253 under control of the T7 promoter. (A) Cells were grown to an OD_{600} of 0.8 and pulse labeled as described in Materials and Methods. Lane 1, *P. putida* WCS358 (pJR253); 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_{600} 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.
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 *xcpA* mutant, which does not assemble pili, grows as a compact, dome-shaped colony. Introduction of the *pilD* (*xcpA*) gene of *P. putida* in the *xcpA* mutant resulted in wild-type colony morphology (Fig. 2). The presence or absence of the retractable pili was also analyzed by using the *P. aeruginosa* pilus-specific phage PO4. The *xcpA* mutant was resistant, whereas the wild type and the *xcpA* 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* *xcpA* mutant.

**Nucleotide sequence analysis.** The 10-kb HindIII fragment of pPX581, 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 preplins; 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).

**FIG. 2.** Plate assay demonstrating elastase secretion. *P. aeruginosa* PAO222 (Xcp+ Pil+) and preplin peptidase mutant KS904 (*xcpA*) 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 *xcpA* mutant carrying vector pLAFR3 compared with the sizes of the other colonies, which are more spread, probably because of twitching motility.
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.

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 pili 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 methylethionine (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 StuI sites were used for subcloning of pilD under control of the lac 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 rRNA (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 rRNA (CGU) gene, indicated in boldface type, starts at nucleotide 702 and ends at nucleotide 777. Underlined nucleotides upstream of the rRNA gene indicate the rRNA promoter (23). Horizontal arrows indicate an inverted repeat.
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 pilated 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 pilated PAO strain. These observations might be explained by the fact that the type IV pilus are retractile by disassembly of the pilus. In the normally pilated 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.

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 *pIC* 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 *pILC* and *pILB*, 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 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-N16-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 exoproto- teins 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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