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Characterization of Genes Required for Pilus Expression in *Pseudomonas syringae* Pathovar phaseolicola

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Nonpiliated, phage ϕ6-resistant mutants of *Pseudomonas syringae* pv. phaseolicola were generated by Tn*5* transposon mutagenesis. A *P. syringae* pv. phaseolicola LR700 cosmid library was screened with Tn*5*-containing *Eco*RI fragments cloned from nonpiliated mutants. The cosmid clone pVK253 complemented the nonpiliated mutant strain HB2.5. A 3.8-kb sequenced region spanning the Tn*5* insertion site contained four open reading frames. The transposon-inactivated gene, designated *pilP*, is 525 bp long, potentially encoding a 19.1-kDa protein precursor that contains a typical membrane lipoprotein leader sequence. Generation of single mutations in each of the three remaining complete open reading frames by marker exchange also resulted in a nonpiliated phenotype. Expression of this gene region by the T7 expression system in *Escherichia coli* resulted in four polypeptides of approximately 39, 26, 23, and 18 kDa, in agreement with the sizes of the open reading frames. The three genes upstream of *pilP* were designated *pilM* (39 kDa), *pilN* (23 kDa), and *pilO* (26 kDa). The processing of the PilP precursor into its mature form was shown to be inhibited by globomycin, a specific inhibitor of signal peptidase II. The gene region identified shows a high degree of homology to a gene region reported to be required for *Pseudomonas aeruginosa* type IV pilus production.

Pseudomonas syringae is an opportunistic plant pathogenic bacterial species divided into pathovars according to the plant species infected. The majority of described *P. syringae* strains are plant pathogenic, but nonpathogenic strains have also been isolated (29). *P. syringae* strains are known to be efficient colonizers of leaf surfaces (reviewed in reference 15), and some strains of *P. syringae* are among the most efficient and common ice-nucleating bacteria (23).

P. syringae pv. phaseolicola causes halo blight in bean plants. *P. syringae* pv. phaseolicola HB10Y produces proteinaceous appendages that were originally identified by their role as bacteriophage ϕ6 receptors (2, 44). In an in vitro adsorption assay, it was shown that the ϕ6-specific pilus of various *P. syringae* pathovars functions in the adsorption of bacterial cells to plant surfaces of both host and nonhost plants (35). The ability to generate symptoms correlated with the presence of pili when bean plants were spray inoculated (34). The pathovars phaseolicola and syringae show different patterns of adsorption: pv. phaseolicola adsorbs mainly to the stomata, while pv. syringae does not show any specific localization in adsorption (35).

Pili (fimbriae) are nonflagellar, filamentous protein appendages assembled on the bacterial cell surface. They are composed of one major subunit, pilin, and often from one to three different kinds of minor subunits that are located at the tip of the filament. The minor subunits can act as lectins that mediate attachment specifically to carbohydrates (13 and references therein), but attachment can also be mediated via protein-protein interactions (46). Genes required for the production of various types of pili are found in one or several gene clusters, where most of the genes have ancillary functions, such as processing, assembly, or regulation of pilus expression (reviewed in references 13, 17, and 40).

About 60% of *P. syringae* isolates act as hosts for bacteriophage ϕ6 (33, 35). Two kinds of spontaneous ϕ6-resistant *P. syringae* pv. phaseolicola mutant phenotypes have been identified. The first class of mutants do not produce pili, and no phage adsorption is observed. The second type of mutants, referred to as superpiliated, adsorb phage more efficiently than the wild type. These strains, however, are resistant to infection by phage ϕ6, apparently because of the inability of the bacterium to retract the pili (33). This resembles the mechanism in *Pseudomonas aeruginosa* PAK, for which the inability of the bacterium to retract the type IV pili results in higher numbers of pili on the bacterial cell surface (19).

In this study, nonpiliated mutants of *P. syringae* pv. phaseolicola HB10Y were generated by Tn*5* mutagenesis. Production of pili was assayed by phage ϕ6 adsorption. A cosmid clone that complemented the nonpiliated mutant strain HB2.5 was isolated. The transposon-inactivated gene *pilP* and three open reading frames (ORFs) upstream were characterized. These four genes show high homology to recently identified *P. aeruginosa* genes *pilM*, *pilN*, *pilO*, and *pilP*, which have been shown to be localized adjacent to the earlier identified *pilQ* gene (24, 25). Inactivation of chromosomal *pilM*, *pilN*, and *pilO* genes with an antibiotic resistance-encoding cassette abolishes pilus production. Possible functions of these genes in *P. syringae* pilus production are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strain HBr, a spontaneous Rif^r derivative of *P. syringae* pv. phaseolicola HB10Y, was used as the piliated, phage ϕ6-sensitive strain. A cosmid library containing *P. syringae* pv. phaseolicola LR700 DNA (10) was obtained from Dallice Mills (Oregon State University), and ^{14}C-labeled bacteriophage ϕ6 was obtained from Dennis Bamford (University of Helsinki).

P. syringae pv. phaseolicola was grown in King's medium B (KB) (22) at 28°C with shaking. Whenever phage ϕ6 was used, cultures were incubated at room temperature (21 to 23°C). *Escherichia coli* was grown in Luria-Bertani (LB) medium (36) at 37°C unless otherwise stated. Antibiotic concentrations were as follows: ampicillin, 150 μg/ml; kanamycin, 25 μg/ml; rifampin, 75 μg/ml; chloramphenicol, 35 μg/ml; and tetracycline, 10 μg/ml for cosmids and 15 μg/ml for

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
E. coli		
DH5α	*hsdR recA lacZYA* ϕ80 *lacZ*ΔM15	Bethesda Research Laboratories
DH5α MCR	*hsdR recA lacZYA* ϕ80 *lacZ*ΔM15 *mcrA*	Bethesda Research Laboratories
P. syringae pv. phaseolicola		
HB10Y	Wild type, $\phi6^s$	44
HBr	HB10Y derivative, Rif^r Pil^+ $\phi6^s$	This study
HB2.5	Tn*5* mutant of HBr, Km^r Rif^r Pil^- $\phi6^r$	This study
MP0.16	Spontaneous mutant of HB10Y, Pil^- $\phi6^r$	33
HB*pilM*::Km	Km cassette inserted in *Not*I site at position 1557, Pil^- $\phi6^r$	This study
HB*pilN*::Km	Km cassette inserted in *Pst*I site at position 2438, Pil^- $\phi6^r$	This study
HB*pilO*::Km	Km cassette inserted in *Apa*I site at position 2839, Pil^- $\phi6^r$	This study
HB2.5.31	Cosmid-complemented HB2.5, Pil^+ $\phi6^s$	This study
Plasmids		
pUC18/19	Ap^r cloning vector	49
pUC4K	Ap^r Km^r, derivative of pUC4 containing ca. 1.3-kb kanamycin resistance cassette	Pharmacia
pBluescript-KS/SK+	Ap^r *lacZ′*, T7 promoter	Stratagene
pGS9	Suicide vector containing Tn*5*; Km^r Cm^r	37
pGP1-2	Km^r; thermoinducible T7 polymerase gene	42
pRK415	Tc^r broad-host-range cloning vector	21
pRK2013	Km^r conjugation helper plasmid	11
pMOB	Ap^r plasmid vector for sequencing	39
pUER2.5	17.7-kb *Eco*RI fragment containing Tn*5* in pUC18	This study
pUC25BN	3.8-kb *Bam*HI-*Nsi*I fragment in pUC18	This study
pVK253	Cosmid complementing mutant HB2.5, Tc^r	This study
pRK25E	12-kb *Eco*RI fragment from pVK253 in pRK415	This study
pRK25EΩ	Sp^r/Sm^r pRK25E derivative with the Ω fragment (30) cloned into the *Eco*RI fragment *Bam*HI site	This study
pRK25BE	10-kb *Bam*HI-*Eco*RI from pRK25E fragment in pRK415	This study
pRK25BN	3.8-kb *Bam*HI-*Nsi*I fragment from pRK25BE in pRK415	This study
pRK25BP	2.4-kb *Bam*HI-*Pst*I fragment in pRK415	This study
pRK25PN	1.4-kb *Pst*I-*Nsi*I fragment in pRK415	This study
pRK25HN	pRK25BN derivative with *Hin*dIII deletion	This study
pRK25PA	pRK25BN derivative with *Pst*I-*Apa*I deletion	This study
pRK25BBS	3.3-kb *Bam*HI-*Bsr*BI fragment from pUC25BN in pRK415	This study
pKSAN	2.9-kb *Acc*I-*Nsi*I fragment in pBluescript pKS+	This study
pSKAN	2.9-kb *Acc*I-*Nsi*I fragment in pBluescript pSK+	This study
pKSSN	1.9-kb *Sac*I-*Nsi*I fragment in pBluescript pKS+	This study
pKSPN	1.3-kb *Pst*I-*Nsi*I fragment in pBluescript pKS+	This study
pKS*pilP*	0.7-kb *Age*I-*Nsi*I fragment in pBluescript pKS+	This study

[a] Abbreviations: Ap^r, ampicillin; Rif^r, rifampin; Km^r, kanamycin; Tc^r, tetracycline; Cm^r, chloramphenicol; Sp^r/Sm^r, spectinomycin/streptomycin resistance. Pil^+, piliated; Pil^-, nonpiliated; $\phi6^s$, phage ϕ6 sensitive; $\phi6^r$, phage ϕ6 resistant.

pRK415 derivatives. In KB medium, the tetracycline concentration was doubled to compensate for the inhibition by the high magnesium concentration. Globomycin was a generous gift from M. Inukai (Biomedical Research Laboratories, Sankyo Co. Ltd.).

Conjugation procedures. Transposon mutagenesis of *P. syringae* pv. phaseolicola was done by conjugation with the Tn*5*-containing suicide plasmid pGS9 (37). Bacterial cells from 5 ml of exponential-growth-phase cultures were pelleted and resuspended in 1 ml of LB broth (2×10^9 CFU/ml). Equal volumes of donor and recipient cell suspensions were mixed, and 100 μl of the mixture was pipetted onto LB agar. The plates were incubated at 37°C for 3 to 4 h and then removed to room temperature (21 to 23°C). After an overnight incubation, bacteria were resuspended in 1 ml of KB broth, and 100-μl samples were plated on KB agar with appropriate selection.

Transfer of pRK415 derivatives was performed by triparental mating, with pRK2013 in *E. coli* HB101 as a helper plasmid (11).

Adsorption assay. The presence of pili was assayed by a phage adsorption assay essentially as described by Romantschuk and Bamford (33). Bacterial strains were grown overnight in KB broth with appropriate selection. A 1-ml amount of the cultures was pelleted and resuspended in 0.5 ml of LB broth (1.0×10^9 to 2.5×10^9 CFU/ml) and incubated with shaking for 20 min at 21°C. ^{14}C-labeled phage was added at a multiplicity of 0.1 to 0.5 (2,000 to 5,000 cpm), and 200-μl aliquots were withdrawn after 20 min of incubation with shaking. Cells were pelleted, and the radioactivity of the pellet and supernatant was measured on an LKB liquid scintillation counter.

DNA manipulations. Plasmid and cosmid DNAs were extracted by the alkaline lysis method (36) and purified on Qiagen columns (Diagen GmbH). Standard techniques were used for enzymatic manipulations and electrophoresis (36). GeneClean (Bio 101 Inc.) was used for purification of DNA fragments from agarose gels. For Southern (38) and colony blotting, Hybond N (Amersham) was used as the support. Probes were labeled with [α-^{32}P]dCTP (Amersham) by nick translation or random priming (36). Hybridization was performed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) at 65°C, and stringent washes were done according to the instructions supplied by the membrane manufacturer.

Generation of marker exchange mutations. Marker exchange mutations were generated as described by Bonas et al. (4). Derivatives of plasmid pRK25BN with the kanamycin cassette from pUC4K inserted in the *Not*I site in the *pilM* gene, the *Pst*I site in the *pilN* gene, or the *Apa*I site in the *pilO* gene were conjugated into strain HBr by triparental mating as described above. Transconjugant colonies were streaked on KB plates containing rifampin and kanamycin for 10 passages. Single colonies were selected and grown with 4 mM D-cycloserine for enrichment of tetracycline-sensitive colonies (43). Dilutions from the enrichment cultures were plated on KB supplemented with rifampin and kanamycin. Plates were incubated at 28°C for 2 days, after which approximately 50 colonies were tested for tetracycline sensitivity. Tetracycline-sensitive clones were confirmed to contain the proper marker exchange mutation by Southern hybridization of total DNA restricted with appropriate enzymes.

DNA sequencing and analysis. A nested set of deletions of the fragment to be sequenced were generated by using the Nested Deletion Kit (Pharmacia). A transposon-based deletion strategy with the TN1000 kit (Gold Biotechnology

Inc.) was also performed as described by Strathmann et al. (39). DNA sequencing was carried out by the dideoxy method with the AutoRead kit (Pharmacia). Sequencing reactions were run on an automated DNA sequencer (A.L.F.; Pharmacia). The sequences were assembled with the Staden Package program (6) on a Sun workstation.

Sequence analysis was done with the Genetics Computer Group (GCG) (8) and PC-GENE (GENOFIT SA) program packages. Homologies to the sequenced DNA and translated ORFs were searched at the National Center for Biotechnology Information with the BLAST network service (1).

Protein expression. Proteins were expressed by a coupled T7 RNA polymerase-promoter gene expression system. Fragments used for the protein expression were cloned in pBluescript-KS or -SK (Stratagene) in *E. coli* and transformed into *E. coli* DH5α containing a thermoinducible T7 polymerase on plasmid pGP1-2 (42). Strains were grown in M9 medium supplemented with 0.2% (vol/vol) glycerol, 0.001% (wt/vol) thiamine, and a 0.2 mM mixture of 18 amino acids. Proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. When used, globomycin in methanol was added to 100 μg/ml 20 min prior to labeling. To negative controls, methanol alone at a final concentration of 1% (vol/vol) was added.

Nucleotide sequence accession number. The DNA sequence determined in this work was submitted to GenBank under accession number L28837.

RESULTS

Tn*5* mutagenesis and isolation of nonpiliated mutants. Following Tn*5* mutagenesis with the suicide plasmid pGS9 as described in Materials and Methods, ϕ6-resistant mutants were isolated from 12 independent conjugations by directly plating bacterial suspensions incubated with phage or by replica plating the colonies onto plates seeded with phage. Phage-resistant clones were tested by cross-streaking them over lines of ϕ6 phage on KB agar plates. Ninety-two clones whose growth was unaffected by the phage were tested by the phage adsorption assay with ^{14}C-labeled phage. Phage was added at a multiplicity of 0.1 to 1.0, which has been shown to give the most efficient adsorption of phage to the pilus receptor and the greatest difference between piliated and nonpiliated bacteria (28). Of the tested strains, 72 were nonadsorbing, with a typical adsorption rate of less than 10% of the added radioactivity, while the same value for the wild-type strain HBr was between 60 and 70%. The phage resistance of the nonadsorbing clones was also confirmed by the inability of ϕ6 to produce plaques on a lawn of the tested strain. To demonstrate the presence of Tn*5*, chromosomal DNA of the mutants was digested with *Eco*RI, which does not cut within the Tn*5* sequence, and subjected to Southern hybridization with pGS9 DNA as the probe. In each case, a single hybridizing band resulted for each of the Tn*5* mutants (data not shown).

Further hybridization experiments divided the mutants into seven groups, each carrying Tn*5* in a different *Eco*RI fragment. The finding of two or more mutants from separate mutagenesis experiments that harbored the transposon within the same *Eco*RI fragment was considered an indication that the nonpiliated phenotype resulted from a mutation induced by the transposon. One mutant, HB2.5, contained a Tn*5* insertion present in a number of independently isolated mutants. Chromosomal DNA was prepared from mutant HB2.5 and digested with *Eco*RI. After gel purification of fragments of more than 10 kb, the DNA was ligated to *Eco*RI-digested pUC18, and ampicillin- and kanamycin-resistant transformants were selected, resulting in the isolation of pUER2.5.

Isolation of complementing cosmid clones. A cosmid library of *P. syringae* pv. phaseolicola LR700 DNA in *E. coli* HB101 was screened by probing colony blots with plasmid pUER2.5. Two cosmids, designated pVK253 and pVK258, which restored ϕ6 sensitivity and phage adsorption to HB2.5 were isolated. The two cosmids were shown to span the same chromosomal region by restriction digests and hybridization. Hybridization of total DNA from *P. syringae* pv. phaseolicola strains HBr and LR700 with pVK253 gave identical hybridization patterns, showing that no rearrangements had occurred in this region between the two strains. In strain HB2.5, the hybridizing *Eco*RI fragment was approximately 5.7 kb larger than in the wild-type strain, as expected if it contained Tn*5*.

Cosmid pVK253, carrying an approximately 22-kb insert, did not complement mutations resulting from Tn*5* insertions in different *Eco*RI fragments, suggesting that these mutations are not located immediately adjacent to the fragment mutated in HB2.5.

Localization of the mutation in HB2.5. Restriction analysis of pUER2.5, containing the mutated *Eco*RI fragment from strain HB2.5, was used to determine the location of the transposon and as an aid in the cosmid subcloning process. Hybridization of an *Eco*RI digest of cosmid pVK253, using the Tn*5*-containing *Eco*RI fragment in pUER2.5 as the probe, resulted in the detection of a 12-kb fragment. This fragment and subclones were cloned into the mobilizable broad-host-range vector pRK415 to test for complementation of HB2.5 (Fig. 1). Plasmids pRK25E (containing the entire *Eco*RI fragment), pRK25BE (containing a 10-kb *Bam*HI-*Eco*RI fragment), and pRK25BN (containing a 3.8-kb *Bam*HI-*Nsi*I fragment) complemented the mutation in HB2.5. As complementation occurred independently of the fragment orientation in relation to the vector-encoded *lac* promoter (Fig. 1), this suggests that the gene responsible for complementation is expressed from an internal promoter contained within the 3.8-kb *Bam*HI-*Nsi*I fragment in pRK25BN.

Nucleotide sequence analysis of the complementing 3.8-kb fragment. The nucleotide sequence of the 3.8-kb *Bam*HI-*Nsi*I fragment in pRK25BN was determined and analyzed for potential protein-coding regions (Fig. 2). The exact site of insertion of Tn*5*, between bases 3658 and 3659, was determined by sequencing the junction between the transposon and the mutated region cloned in pUER2.5. The transposon interrupts a 525-bp ORF starting at base 3166 and ending after base 3690. The fragment contains three additional ORFs upstream and one partial reading frame downstream of the transposon-containing ORF. The termination codons of each of these ORFs overlap the start codons of the downstream ORF, suggesting that they might form a single transcriptional unit. The 5′ end of the fragment appears to encode a partial ORF transcribed in the opposite direction (Fig. 2).

The transposon-inactivated ORF, designated *pilP*, potentially encodes a 19.1-kDa protein of 175 residues. The first 18 deduced amino acids of the PilP sequence have characteristics of typical signal sequences of lipoproteins, with the consensus tetrapeptide LAGC at the junction between the signal sequence and the mature peptide (47). The mature, putative 17.3-kDa lipoprotein shows 70% identity and 85% similarity to the deduced *pilP* gene product, recently suggested to be required for type IV pilus production in *P. aeruginosa* (accession number U12892 [25]).

ORF1, the leftmost ORF in the putative operon, is 1,062 bp long and potentially encodes a polypeptide of 354 residues (38 kDa). The ORF1 product is predicted to contain two membrane-spanning segments at the C-terminal part of the protein (Fig. 2), as determined by the method of Rao and Argos (32). At the protein level, it has 85% identity and 93% similarity to the recently identified PilM protein of *P. aeruginosa*, and it also has limited homologies with FtsA of *Bacillus subtilis* (48% similarity) and *E. coli* (44% similarity), a protein required for cell division (3, 50).

ORF2 is 596 bp long, potentially encoding a polypeptide 199 residues in length (22 kDa) containing one putative N-terminal membrane-spanning segment (Fig. 2). Apart from the high

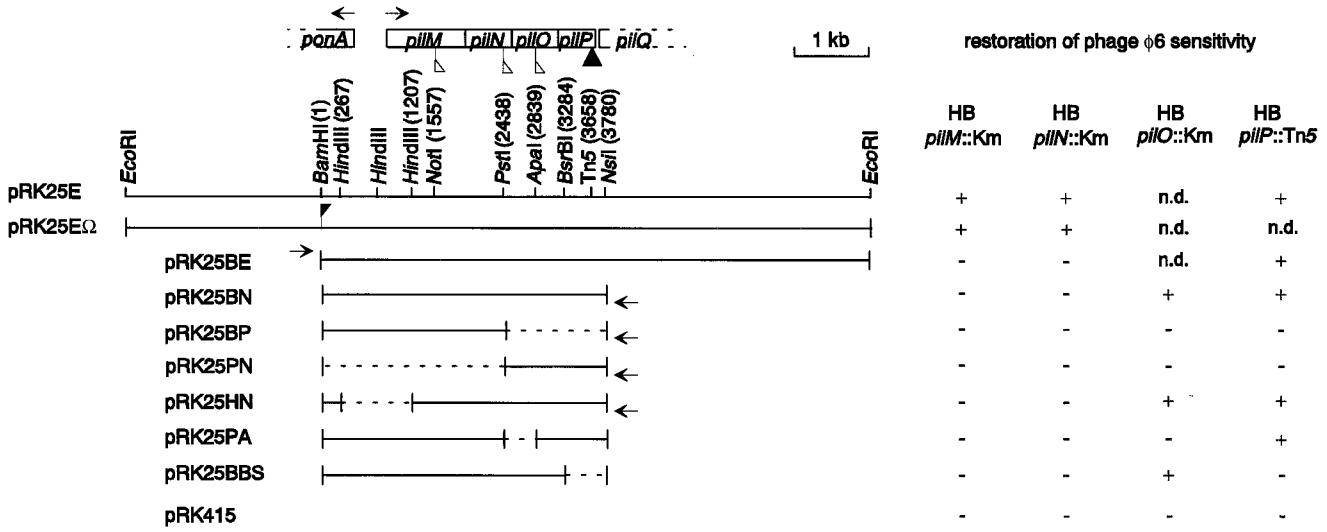

FIG. 1. Genetic organization and restriction map of the 3.8-kb *Bam*HI-*Nsi*I fragment in relation to the 12-kb *Eco*RI-*Eco*RI and 10-kb *Bam*HI-*Eco*RI fragments. Putative genes coded by the sequenced fragment are shown with boxes. Restriction sites used for cloning of different constructs as well as for insertions of the kanamycin cassette are shown. The nucleotide positions of the insertion sites are shown in parentheses. Restoration of phage ϕ6 sensitivity to different phage-resistant, nonpiliated mutants by the constructs is shown on the right. Horizontal arrows above the putative genes indicate the direction of transcription, deduced from the sequence data, and those above different clones indicate the direction of transcription initiated from the *lac* promoter in the vector. Symbols: ◤, insertion site for Ω fragment (30) in plasmid pRK25EΩ; ◺, insertion site for kanamycin resistance cassette marker-exchanged into the chromosome; ▲, insertion site for Tn*5*; n.d., not determined.

homology (70% identity and 78% similarity) with the PilN protein of *P. aeruginosa*, there is also good regional homology (29% identity and 55% similarity), limited to the C-terminal half, with the PefL protein of *Xanthomonas campestris* (accession number L02630), reported to be required for protein export.

ORF3, a 620-bp-long ORF, potentially encodes a polypeptide of 207 residues (23 kDa) (Fig. 2) which also contains one putative N-terminal membrane-spanning segment. The deduced amino acid sequence has significant homology to PilO of *P. aeruginosa* (75% identity and 83% similarity).

The partial ORF at the 5′ end of the determined sequence shows a high degree of nucleic acid sequence homology with the 5′ end of the *ponA* gene of *P. aeruginosa* (accession number L13867 [24]) and *E. coli* (20). The 3′ end of the fragment ends in what appears to be the coding sequence for the N terminus of the *P. syringae* homolog of the *P. aeruginosa pilQ* gene, which is also required for type IV pilus production in *P. aeruginosa* (24).

Complementation of mutations in *pilM-O* genes. To determine if the genes within the sequenced gene cluster are required for *P. syringae* pilus biogenesis, marker exchange mutations within ORF1, ORF2, and ORF3, hereafter designated *pilM*, *pilN*, and *pilO*, respectively, were generated as described in Materials and Methods. The insertion of a kanamycin resistance cassette into each of the cloned genes and recombination into the chromosome (resulting in strains HB*pilM*::Km, HB*pilN*::Km, and HB*pilO*::Km) abolished phage sensitivity and phage adsorption in all three cases, showing that each is needed for pilus production by *P. syringae* pv. phaseolicola HBr.

To study the transcriptional organization of the potential gene cluster, plasmid pRK25BN and plasmids containing subclones of the *Bam*HI-*Nsi*I fragment were used to test for complementation of each of the insertion mutations. The complementation results are summarized in Fig. 1 and suggest that *pilM*, *-N*, *-O*, and *-P* are organized into two groups that may represent transcriptional units. The *pilM* and *pilN* mutants were not complemented by the 3.8-kb *Bam*HI-*Nsi*I fragment in pRK25BN or any of its subclones. The 12-kb *Eco*RI fragment in plasmid pRK25E, containing 2.6 kb upstream of the *Bam*HI site, complemented these mutants. So did plasmid pRK25EΩ, with an interposon (30) cloned into the *Bam*HI site of the *Eco*RI fragment of pRK25E, showing that the promoter required for *pilMN* expression is located downstream of the *Bam*HI site. The failure of pRK25BN to complement *pilMN* indicates a requirement for regions upstream of the *Bam*HI site for expression of these genes.

Mutations in *pilO* and *pilP*, on the other hand, were complemented by pRK25BN and certain subclones of it (Fig. 1). As expected, the *pilO* mutant was not complemented by pRK25PA, a construct with a deletion from the *Pst*I site (2438) to the *Apa*I site (2839), removing part of the putative *pilO* gene. The *pilP* mutant resulting from the Tn*5* insertion could not be complemented with the pRK25BBS, in which the *pilP* 3′ sequence is deleted, starting from the *Bsr*BI site after base 3284. The 1.4-kb *Pst*I-*Nsi*I fragment could not complement *pilO* and *pilP* mutants despite spanning those two putative genes. Plasmid pRK25HN, containing a 940-bp *Hin*dIII-*Hin*dIII deletion (bases 267 to 1207) of the N-terminal portion of the 3.8-kb fragment, was able to complement both *pilO* and *pilP* mutations. Taken together, the results suggest the presence of a functional promoter between bases 1207 and 2438, apparently within the putative *pilN* gene.

Expression of proteins encoded by clone pRK25BN. To determine if the *pilM-P* ORFs expressed appropriately sized proteins, plasmid subclones of the sequenced region were tested in the T7 expression system described by Tabor and Richardson (42). Clones containing *pilM-P* within a 2.9-kb *Acc*I-*Nsi*I fragment (pSKAN), *pilN-P* within a 1.9-kb *Sac*I-*Nsi*I fragment (clone pKSSN), *pilO* and *pilP* within a 1.3-kb *Pst*I-*Nsi*I fragment (clone pKSPN), and the *pilP* gene alone within a 698-bp *Age*I-*Nsi*I fragment (pKS*pilP*) were each tested. As controls, a plasmid containing all four genes in the opposite orientation (pKSAN) to the T7 promoter as well as the vector pBluescript-KS and -SK plasmids were used (Fig. 3).

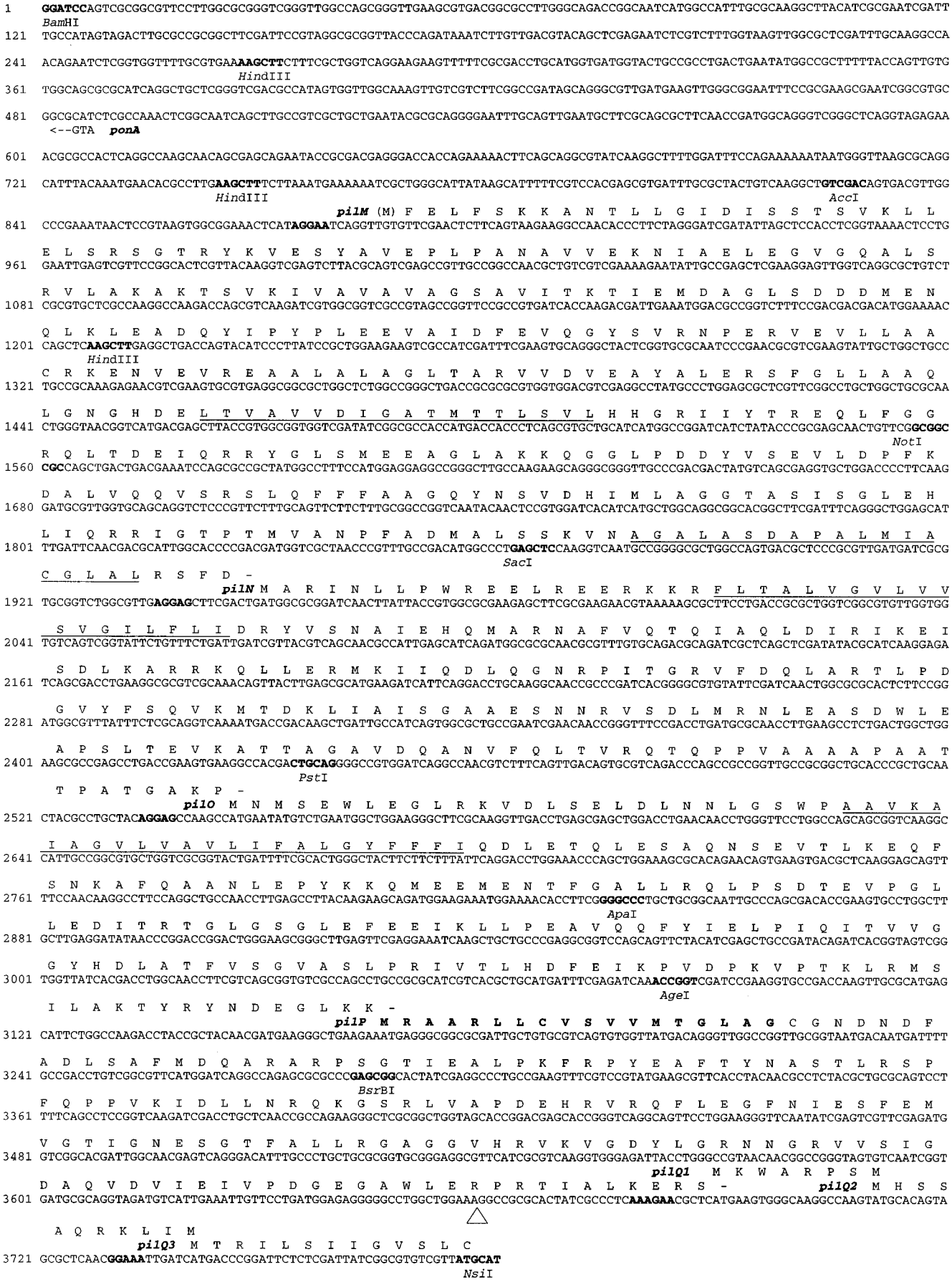
BamHI
HindIII
<--GTA ponA
pilM (M) F E L F S K K A N T L L G I D I S S T S V K L L
HindIII
AccI
E L S R S G T R Y K V E S Y A V E P L P A N A V V E K N I A E L E G V G Q A L S
R V L A K A K T S V K I V A V A V A G S A V I T K T I E M D A G L S D D D M E N
Q L K L E A D Q Y I P Y P L E E V A I D F E V Q G Y S V R N P E R V E V L L A A
HindIII
C R K E N V E V R E A A L A L A G L T A R V V D V E A Y A L E R S F G L L A A Q
L G N G H D E L T V A V V D I G A T M T T L S V L H H G R I I Y T R E Q L F G G
NotI
R Q L T D E I Q R R Y G L S M E E A G L A K K Q G G L P D D Y V S E V L D P F K
D A L V Q Q V S R S L Q F F F A A G Q Y N S V D H I M L A G G T A S I S G L E H
L I Q R R I G T P T M V A N P F A D M A L S S K V N A G A L A S D A P A L M I A
SacI
C G L A L R S F D -
pilN M A R I N L L P W R E E L R E E R K K R F L T A L V G V L V V
S V G I L F L I D R Y V S N A I E H Q M A R N A F V Q T Q I A Q L D I R I K E I
S D L K A R R K Q L L E R M K I I Q D L Q G N R P I T G R V F D Q L A R T L P D
G V Y F S Q V K M T D K L I A I S G A A E S N N R V S D L M R N L E A S D W L E
A P S L T E V K A T T A G A V D Q A N V F Q L T V R Q T Q P P V A A A A P A A T
PstI
T P A T G A K P -
pilO M N M S E W L E G L R K V D L S E L D L N N L G S W P A A V K A
I A G V L V A V L I F A L G Y F F F I Q D L E T Q L E S A Q N S E V T L K E Q F
S N K A F Q A A N L E P Y K K Q M E E M E N T F G A L L R Q L P S D T E V P G L
ApaI
L E D I T R T G L G S G L E F E E I K L L P E A V Q Q F Y I E L P I Q I T V V G
G Y H D L A T F V S G V A S L P R I V T L H D F E I K P V D P K V P T K L R M S
AgeI
I L A K T Y R Y N D E G L K K -
pilP M R A A R L L C V S V V M T G L A G C G N D N D F
A D L S A F M D Q A R A R P S G T I E A L P K F R P Y E A F T Y N A S T L R S P
BsrBI
F Q P P V K I D L L N R Q K G S R L V A P D E H R V R Q F L E G F N I E S F E M
V G T I G N E S G T F A L L R G A G G V H R V K V G D Y L G R N N G R V V S I G
pilQ1 M K W A R P S M
D A Q V D V I E I V P D G E G A W L E R P R T I A L K E R S - pilQ2 M H S S
A Q R K L I M
pilQ3 M T R I L S I I G V S L C
NsiI

FIG. 2. Nucleotide sequence of the 3.8-kb fragment complementing nonpiliated mutant strain HB2.5. The Tn*5* insertion site after base 3658 is indicated by a triangle below the sequence. Potential ribosome-binding sites and the PilP signal sequence are shown in boldface. Possible initiation codons are indicated for the 5′ *pilQ* ORF. Restriction sites used in the subcloning for complementation, protein expression, and gene inactivation studies are indicated. Potential membrane-spanning segments of the polypeptides, determined by the method of Rao and Argos (32), are underlined.

The expression of pSKAN resulted in four insert-specific labeled proteins of approximately 39, 26, 23, and 18 kDa (Fig. 3, lane 5). The fragment in plasmid pKSSN (Fig. 3, lane 6) expressed the 26- and 18-kDa proteins at levels comparable to those expressed from pSKAN, whereas the 23-kDa protein band was substantially reduced in intensity but still visible. As expected, the 39-kDa band was absent. The 26-kDa and 18-kDa polypeptides were also expressed by pKSPN (Fig. 3, lane 7). In all lanes with a visible 18-kDa band, a faint 20-kDa band could also be observed. When *pilP* was expressed alone from the T7 promoter (clone pKS*pilP*), only one band of approximately 19 kDa was observed. Expression from pKSAN (Fig. 3, lane 4) resulted in one poorly expressed band, specific to this clone, of an intensity that was not substantially higher than the background expression level observed in the other clones.

Since the deduced amino acid sequence of the PilP protein suggested that it was synthesized as a potential precursor processed by the lipoprotein signal peptidase II, globomycin, a specific signal peptidase II inhibitor (18), was used to test for the inhibition of PilP processing in clones in which the protein appeared in its processed 18-kDa form (Fig. 4). In all reactions with mixtures supplemented with globomycin 20 min prior to labeling, most of the PilP was expressed in a higher-molecular-mass 20-kDa form, suggesting that inhibition of signal peptide cleavage by signal peptidase II had occurred. The same clones supplemented with methanol alone (used as the globomycin solvent) expressed only the 18-kDa form of PilP. Globomycin treatment had no effect on any of the other expressed proteins.

DISCUSSION

This study was undertaken to characterize genes involved in the production of the phage ϕ6-specific pilus of *P. syringae* pv. phaseolicola HB10Y. Tn*5* insertion mutants could be divided into seven groups, each harboring the transposon in different *Eco*RI fragments, which may reflect a relatively high number of genes required for proper expression of the pilus on the bacterial cell surface. Although some of these fragments may originate from the same gene cluster, it seems obvious that proper expression of the ϕ6-specific pilus requires genes from more than one gene cluster. Such complexity has been described for the type IV pili expressed by *P. aeruginosa* and several other animal pathogens (reviewed in references 16 and 40). Indeed, the phage ϕ6-sensitive strain DC3000 of *P. syringae* pv. tomato has been shown to produce type IV pili (27).

In this report, we have demonstrated that at least four genes, *pilM*, *pilN*, *pilO*, and *pilP*, are required for pilus biogenesis by *P. syringae*. Each of the four genes was shown to be required by either the insertion of Tn*5* (into the *pilP* gene) or the introduction of a kanamycin resistance cassette (*pilM-O*), which rendered the mutant bacterium nonpiliated and phage resistant. In addition, from the homology of the DNA sequence 3′ to the *pilP* gene with the *P. aeruginosa pilQ* gene, it appears likely that a *P. syringae pilQ* homolog is also located within the same gene cluster. The *P. aeruginosa pilQ* gene has previously been shown to be required for type IV pilus production and to encode a protein that belongs to a class of outer membrane proteins involved in protein secretion and DNA uptake (16, 24). The presence of a functional *P. syringae pilQ* gene is also supported by the observation that plasmid pRK25BE, containing an approximately 4-kb region downstream of the *pilP* gene, complemented a spontaneous nonpiliated, ϕ6-resistant mutant, MP0.16, while plasmid pRK25BN, containing only the N terminus of the putative *P. syringae pilQ* sequence, did not (data not shown). This suggests that the mutation in MP0.16 is located downstream of *pilP* within the *pilQ* gene or an additional, as yet uncharacterized, pilus biogenesis gene further downstream.

Complementation experiments suggest that the *P. syringae pil* genes are potentially transcribed in two units. The first two genes, *pilM* and *pilN*, were complemented by the 12-kb *Eco*RI fragment (pRK25E) but not by the 3.8-kb sequenced region (pRK25BN). A transcriptional terminator inserted in the unique *Bam*HI site of the complementing fragment, generating pRK25EΩ, did not stop complementation, showing that the promoter is located within the sequenced region. However, the 2.6-kb upstream region in pRK25E apparently contains a sequence(s) essential for the expression of genes *pilM* and *pilN*. In fact, a requirement for upstream regulatory sequences has been reported for expression of the *E. coli ftsA* gene (9), the

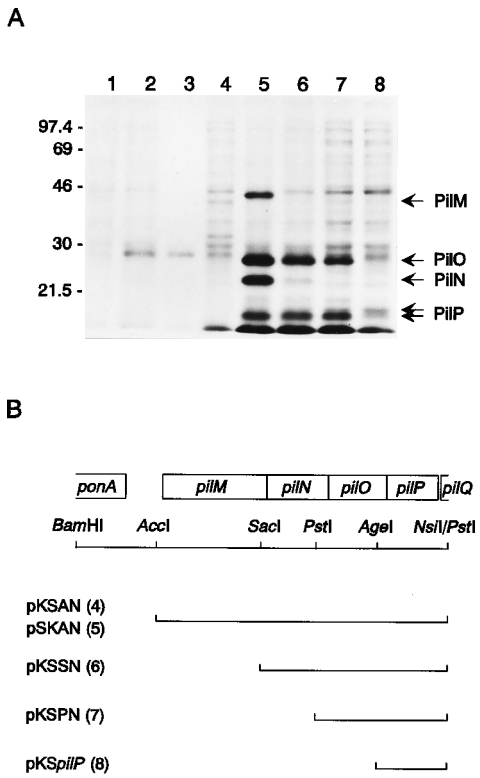

FIG. 3. Protein expression of all four ORFs and deletion derivatives in *E. coli* DH5α. (A) Autoradiogram of the ^{35}S-labeled proteins run on 12% PAGE–SDS gels. The positions of expressed PilM, PilN, PilO, and the precursor and mature forms of PilP are marked with arrows on the right, and those of molecular size standards are marked on the left (in kilodaltons). Lanes: 1, DH5α(pGP1-2); 2, DH5α(pGP1-2, pBluescript-KS); 3, DH5α(pGP1-2, pBluescript-SK); 4, DH5α(pGP1-2, pKSAN); 5, DH5α(pGP1-2, pSKAN); 6, DH5α(pGP1-2, pKSSN); 7, DH5α(pGP1-2, pKSPN); 8, DH5α(pGP1-2, pKS*pilP*). (B) Plasmid constructs used in protein expression experiments. The numbers in parentheses indicate the corresponding lanes in the autoradiogram in panel A.

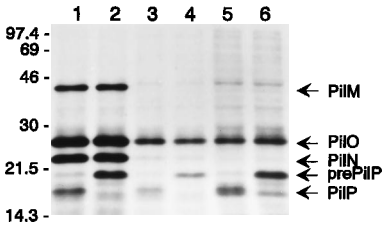

FIG. 4. Autoradiogram of the protein expression and labeling experiment, showing specific inhibition of signal peptidase II processing of PilP prelipoprotein by globomycin. The positions of the molecular size standards are shown on the left (in kilodaltons). Lanes: 1, pSKAN; 2, pSKAN plus globomycin; 3, pKSSN; 4, pKSSN plus globomycin; 5, pKSPN; 6, pKSPN plus globomycin.

gene product with which the PilM protein shows significant homologies. In contrast, *pilO* and *pilP* could be complemented by the 3.8-kb *Bam*HI-*Nsi*I fragment (pRK25BN). A smaller clone, pRK25PN, containing only the coding sequence for the two genes did not complement, however, suggesting that the promoter for expression of *pilO* and *pilP* is located upstream of the *Pst*I site. As clone pRK25HN, containing a deletion from base 247 to base 1207, complemented mutations in both *pilO* and *pilP*, the active promoter in these complementation experiments must be situated somewhere within the *pilN* gene. The T7 promoter expression studies demonstrated that the four genes could be cotranscribed from a promoter upstream of *pilM*, suggesting that transcriptional terminators are not located within this region. No apparent regions of RNA secondary structure were detected within the sequenced DNA, as represented by significant inverted repeats. Also considering the translational overlap of the genes, it is very likely that *pilM-P* are cotranscribed in *P. syringae* pv. phaseolicola, with internal promoters possibly playing a role in the regulation of pilus biogenesis genes.

As mentioned above, the nucleotide sequence of *pilM-P* shows that the termination codon of each ORF overlaps the initiation codon of the following one, suggesting that translational coupling of gene expression may be occurring, as has been observed in many bacterial operons (reviewed in references 12 and 51). Protein expression experiments demonstrated that efficient production of PilN required the expression of the upstream gene *pilM*, pointing to translational coupling of at least these two genes. Alternatively, PilM expression may be required for the stability of the *pilN* gene product. Thus, the involvement of PilM in pilus production may be only indirect.

The deduced amino acid sequence of PilM is predicted to contain at least two potential membrane-spanning segments and shows localized homology to the FtsA protein of *E. coli* and *B. subtilis* as well as homology to a group of proteins identified as possessing a novel ATPase domain, recently identified by Bork et al. (5). As most of the conserved motifs were also found in PilM, it may therefore act as a membrane-bound ATPase, generating energy for a step directly or indirectly related to pilus production. The insertion mutation in the *pilM* gene, however, had no apparent effect on cell septation or division, suggesting that its role is not directly analogous to that of FtsA.

PilN and PilO, as expressed in the T7 expression experiments, were estimated to be 23 and 26 kDa in size, respectively. Each is predicted to contain one membrane-spanning segment near the N terminus of the protein. PilN has some homology to PefL, a protein required for protein secretion by *X. campestris*. PilO, however, does not show significant homology to any known sequence in the data bank except for the *P. aeruginosa* counterpart.

The *pilP* gene, mutated in the initial screen for Tn*5* insertions that affect pilus biogenesis, encodes a 19.1-kDa protein with a typical lipoprotein signal sequence. Cleavage of the PilP signal sequence was shown to be inhibited by the presence of globomycin, an inhibitor of the lipoprotein-specific signal peptidase II (7, 18), demonstrating that PilP is a lipoprotein. When expressed from clones also containing the *pilO* gene, PilP was seen as either an 18-kDa or 20-kDa protein. When expressed alone, however, PilP appeared instead as a 19-kDa form. The processing of diacylglyceryl-modified prolipoproteins by signal peptidase II is postulated to occur at the periplasmic face of the inner membrane (26), and mutations in a number of *sec* genes are known to affect both lipid modification and cleavage of lipoproteins in *E. coli* (14, 41, 45). As signal peptidase II cleaves only modified lipoproteins (47), it might be suggested that the 20-kDa and 18-kDa forms of PilP represent the precursor and cleaved, lipid-modified forms, respectively. The 19-kDa form expressed in the absence of *pilO* may represent an unmodified precursor of PilP, suggesting that PilO is required for proper positioning of PilP in the membrane for lipid modification and subsequent processing to occur.

Lipoproteins can be anchored to either the cytoplasmic or the outer membrane in gram-negative bacteria. In *E. coli*, localization appears to be dependent in part on the amino acid sequence adjacent to the processing site (48). If the same predictive rules apply to *P. syringae* lipoproteins, PilP is likely to be found in the outer membrane, since the cysteine at the signal peptidase II cleavage site is followed by a valine rather than an acidic amino acid, which often defines a cytoplasmic membrane location. This is in agreement with the hypothesis of Martin et al. (25), who have proposed that the PilP of *P. aeruginosa* is associated with an outer membrane pore-like structure composed of the *pilQ* gene product, much in the same fashion as proposed for the association of the lipoprotein PulS with PulD in the pullulanase secretion apparatus (reviewed in reference 31).

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