Characterization of Type IV Pilus Genes in *Pseudomonas syringae* pv. *tomato* DC3000

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Many strains of *Pseudomonas syringae* produce retractile pili that act as receptors for lytic bacteriophage $\phi 6$. As these are also characteristics of type IV pili, it was postulated that *P. syringae* may possess genes for type IV pilus biogenesis. A cosmid clone bank of *P. syringae* pv. *tomato* DC3000 genomic DNA was used to complement a mutant of *Pseudomonas aeruginosa* defective in the PilD (XcpA) prepilin peptidase gene by selection for restoration of extracellular protein secretion, a function also known to require PilD. A cosmid able to complement this mutant was also able to complement mutations in the *pilB* and *pilC* genes, suggesting that, if the organization of these genes is similar to that of *P. aeruginosa*, the cosmid may contain the *P. syringae* *pilA*. This was confirmed by sequencing a region from this plasmid that was shown to hybridize at low stringency to the *P. aeruginosa* *pilA* gene. The deduced *P. syringae* PilA polypeptide possesses the characteristic properties of the type IV pilins. Heterologous expression of the *P. syringae* *pilA* in *P. aeruginosa* was also shown, conferring not only $\phi 6$ phage sensitivity to *P. aeruginosa* *pilA* mutants but also sensitivity to PO4, a lytic bacteriophage specific for the pilus of *P. aeruginosa*. This suggests that additional components might be present in the mature pilus of *P. aeruginosa* that are the true receptors for this phage. Chromosomal mutations in *P. syringae* pv. *tomato* DC3000 *pilA* and *pilD* genes were shown to abolish its sensitivity to bacteriophage $\phi 6$. To determine the importance of *P. syringae* pilus in plant leaf interactions, these mutations were tested under laboratory and field conditions. Although little effect was seen on pathogenicity, culturable leaf-associated population sizes of the *pilA* mutant were significantly different from those of the wild-type parent. In addition, the expression of the DC3000 *pilA* gene appears to contribute to the UV tolerance of *P. syringae* and may play a role in survival on the plant leaf surface.

Pseudomonas syringae is a plant-pathogenic bacterial species causing disease on aerial parts of a wide range of plant

hosts (reviewed in Hirano and Upper 1990). *P. syringae* strains can multiply to high epiphytic population sizes without causing disease symptoms (reviewed in Hirano and Upper 1990). On host plants, however, disease incidence is positively correlated with leaf-associated population size (Lindemann et al. 1984; Rouse et al. 1985).

In previous studies of *P. syringae*, nonpilated and superpilated spontaneous mutants were isolated by their resistance to the pilus-specific phage $\phi 6$ (Romantschuk and Bamford 1985), and the nonpilated mutant strain attached poorly to leaf surfaces (Romantschuk and Bamford 1986). The superpilated phenotype of certain phage-resistant mutants was very reminiscent of the behavior shown by *Pseudomonas aeruginosa* hyperpilated mutants, which are resistant to the pilus-specific phage PO4 (Bradley 1972; Johnson and Lory 1987). These results suggested that pili expressed by *P. syringae* may also be of the type IV class.

Because of their retractile nature, the type IV pili mediate a form of bacterial movement known as twitching motility (Bradley 1980) and have been shown to be important virulence factors in several animal pathogens including *P. aeruginosa*, *Neisseria gonorrhoeae*, *Moraxella bovis*, *Dichelobacter nodosus*, and *Vibrio cholerae* (reviewed in Strom and Lory 1993). Type IV pili have also been found in plant growth promoting *Pseudomonas putida* (de Groot et al. 1994), *Myxococcus xanthus* (Wu and Kaiser 1995), and phytopathogenic *Xanthomonas campestris* (Ojanen-Reuhs et al. 1997; van Doorn et al. 1994). In *X. campestris* pv. *hyacinthi* the type IV pili were shown to attach to the stomata of hyacinth leaves (van Doorn et al. 1994), whereas the type IV pili of *X. campestris* pv. *vesicatoria* did not have a role in attachment to tomato leaves (Ojanen-Reuhs et al. 1997). In the latter case, however, it was shown that pilated bacteria formed microcolonies, aggregations of bacteria, that contribute to UV tolerance.

Identification of the *P. syringae* pv. *phaseolicola* *pilMNOP* gene region, required for production of $\phi 6$ phage receptor pilus, showed that the gene products share high homology and a similar organization to the corresponding genes required for type IV pilus production in *P. aeruginosa* (Martin et al. 1995; Roine et al. 1996). In *P. aeruginosa*, the *pilA* gene encodes prepilin monomers that constitute the major structural subunit of the pilus. PilA contains the unusual amino acid *N*-methylphenylalanine as the first residue of the mature protein, which is produced from prepilin by removal of a short 6- to 7-amino-acid leader sequence (Frost et al. 1978; Nunn and Lory 1991; Strom et al. 1993). Cleavage of the prepilin leader pep-

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Nucleotide and/or amino acid sequence data of *pilA* gene can be found at GenBank under the accession number L35847.

tide by PilD (also known as XcpA) is required for assembly into pili. The assembly of pili also requires the functions of a large number of pilus biogenesis genes, including *pilB* and *pilC*. In *P. aeruginosa* PAK these genes, along with *pilD*, reside in an operon adjacent to and divergently transcribed from *pilA* (Nunn et al. 1990). In addition to its role in pilin biogenesis, PilD is required for the extracellular secretion of several important toxins and hydrolytic enzymes of *P. aeruginosa* (Nunn and Lory 1992; Strom et al. 1991) as it also cleaves and methylates pilin-like proteins (XcpT-W) that are components of the type II protein secretion apparatus.

Because of the likelihood that *P. syringae* contains functional homologues of the *P. aeruginosa pilABCD* genes and the recent development of a direct selection for PilD-dependent protein secretion by *P. aeruginosa* (Kagami et al. 1998), we describe here the complementation of a *P. aeruginosa pilD* mutant with a *P. syringae* cosmid bank, the characterization of the linked *pilA* gene, and its role in the interactions with plant leaf surfaces.

RESULTS AND DISCUSSION

Complementation of a *P. aeruginosa pilD* mutant.

To generate a broad-host-range library of the *P. syringae* genome, 4- to 16-kb partially digested *Sau3AI* genomic fragments of DC3000 were cloned into *Bam*HI-cut pDN18. This library was introduced into the *P. aeruginosa pilD* mutant B30 by conjugation and complementing transconjugants were selected on lipid agar plates as described by Kagami et al. (1998). These plates allowed for the selection of colonies able to utilize olive oil as a source of carbon after its hydrolysis by secreted lipase. The growth of the complementing transconjugants appeared as foci since secretion of lipase results in the growth of cells immediately adjacent to it. In addition, the hydrolysis of the lipid resulted in the release of oleic acid, and gave an intense red coloration surrounding the clones due to the pH sensitivity of the neutral red indicator included in the plates. Colonies exhibiting growth and red coloration were picked at the center of growth and re-streaked to obtain isolated single colonies restored for their ability to export lipase.

After the introduction of the *P. syringae* genomic library, complemented colonies arose at a frequency of approximately 10^{-4} and five independent clones conferring ability to grow on lipid agar were isolated for further characterization. Each of the five clones also restored piliation as determined by phage PO4 sensitivity and colony morphology. In addition, each of the five clones hybridized with a 1-kb *Sal*I fragment containing the *P. aeruginosa pilD* gene used as the probe (data not shown).

Generation of a *P. syringae pilD* mutant.

A 5.6-kb *Kpn*I-*Pst*I fragment, which gave a positive signal with the *P. aeruginosa pilD* probe DNA, was isolated from one of the complementing plasmids and cloned into pDN18. To determine whether this smaller fragment encoded a functional prepilin peptidase, the resulting plasmid, designated pDCD58, was introduced into PAK B30 and was able to restore PilD-dependent protein secretion (growth on lipid agar) and phage sensitivity. Further subcloning and hybridization experiments showed that a *Hind*III site was present within the putative *pilD* gene of *P. syringae* (Fig. 1), as subclones from *Hind*III digests failed to complement, and digestion with

*Hind*III separated a region of homology to the PAK *pilD* probe as seen on a Southern blot (data not shown).

A *pilD* insertion mutant of *P. syringae* DC3000 was generated by marker exchange mutagenesis as described in Materials and Methods, and it was tested for sensitivity to phage ϕ 6 by cross-streaking bacteria over a phage line on an agar plate. The *pilD* insertion mutant of DC3000 was resistant to infection while the wild-type strain was sensitive (data not shown), showing that the *P. syringae pilD* gene is required for the expression of a functional ϕ 6 receptor pilus by this organism.

Cloning of *P. syringae pilA* gene.

The five pDN18 clones able to complement PAK *pilD*-mutant strain B30 were further characterized to determine if they also contained the *P. syringae pilA* gene, by hybridization with a 500-bp *Stu*I-*Cl*aI fragment containing the *pilA* pilin structural gene of *P. aeruginosa*. Two of the five clones, designated pDCDA18 and pDCDA25, hybridized with this probe at low stringency (approximately 70% mismatch). Further hybridization and subcloning of fragments from pDCDA18 demonstrated that a 1.6-kb *Hind*III-*Xba*I fragment contained the *pilA* hybridizing sequences. Nucleotide sequencing of this fragment showed that it contained an open reading frame (ORF) predicted to encode a polypeptide of 143 amino acids (Fig. 2). As seen in Figure 3, the first 33 amino acids show 88% identity to the 149 amino acid PilA protein of *P. aeruginosa* (Sastry et al. 1985). Further sequencing of the region upstream of this ORF revealed a consensus RpoN binding site (GG-N₁₀-GC) that is very similar in sequence and location to that found upstream of *P. aeruginosa pilA* (Johnson et al. 1986). As with *P. aeruginosa*, the calculated G+C content of the *pilA* gene was lower (47%) than the average (59 to 61%) of the *P. syringae* genome. These results confirm the previous results by Roine et al. (1996), which suggested that the *P. syringae* pili acting as receptors for bacteriophage ϕ 6 also belong to the family of type IV pili. Whether the expression of *pilA* in *P. syringae* pv. *tomato* DC3000 and other strains is dependent on RpoN as it is in *P. aeruginosa* (Ishimoto and Lory 1989) remains to be studied.

The PilA of *P. syringae* DC3000 appears to show high homology with the other members of the type IV pilins within the first 35 amino acids, but diverges sharply after this point (Fig. 3). The N-terminal region is thought to be highly conserved because of its role in formation of the pilus multimers (Strom and Lory 1993), and a closer examination of the N terminus of the *P. syringae* PilA revealed that it has the distinguishing characteristics of the type IV prepilins. These are a 6-residue basic leader sequence with glycine at position -1 followed by a phenylalanine residue and a stretch of hydrophobic amino acids with the invariable glutamate residue at position +5. Two tyrosine residues at positions 24 and 27, which are highly conserved in the type IV pilins, are also present. Along the entire polypeptide *P. syringae* pv. *tomato* DC3000 PilA showed highest homology (43% identity, 58% similarity) with *Aeromonas hydrophila* type IV pilus subunit TapA (Pepe et al. 1996).

It has previously been noted that all type IV pilins contain at least two cysteine residues at the C terminus that are thought to form a disulfide stabilized loop structure. Although DC3000 contains two cysteine residues in approximately the same location, their spacing is different from that seen with

most type IV pilins (Fig. 3.), and suggests that the proposed "loop" that these residues form in this organism (Strom and Lory 1993) may be very different in structure. Interestingly, the DC3000 PilA also has another set of cysteine residues (residues 56 and 66) that are conserved in their spacing in the *A. hydrophila* TapA (Pepe et al. 1996), *Escherichia coli* PpdD (Whitchurch and Mattick 1994), *Haemophilus influenzae* PpdD (Fleischmann et al. 1995), and *D. nodosus* class II FimA (Mattick et al. 1991), although the significance of this homology is unclear.

Heterologous expression of the *P. syringae* *pilA* gene in *P. aeruginosa*.

To determine if pDCDA18 was able to direct the assembly of *P. syringae* pv. *tomato* DC3000 pili on the surface of *P. aeruginosa*, this plasmid was mobilized into wild-type PAK and the *pilA* deletion mutant of PAK constructed in this study, PAK Δ A. The expression and assembly of functional *P. syringae* pili on the surface of PAK was tested with bacteriophage ϕ 6. Whereas wild-type PAK and PAK Δ A are both resistant to infection with ϕ 6, both strains became phage sensitive when carrying pDCDA18, suggesting that *P. syringae* type pili were being synthesized and assembled on the cell surface.

Since pDCDA18 contained sequences in addition to *pilA*, a 1.6-kb *Hind*III-*Xba*I fragment encoding only the *P. syringae* *pilA* gene was inserted into the pDN18 to generate plasmid pDCA. Introduction of this plasmid into either wild-type PAK or PAK Δ A also conferred sensitivity to ϕ 6, further demonstrating that the pilin precursor encoded by the *P. syringae* *pilA* gene is functionally homologous to its *P. aeruginosa* counterpart and is efficiently recognized by the *P. aeruginosa* pilus biogenesis apparatus. Surprisingly, introduction of the pDCA into PAK Δ A also generated sensitivity to the *P. aeruginosa* pilus-specific phage PO4 (Fig. 4), whereas the PAK Δ A strain alone or PAK Δ A(pDN18) carrying the vector alone were resistant to this phage. Piliated wild-type *P. syringae* is not itself sensitive to PO4, suggesting that it is not the *P. syringae* PilA subunit that is acting as the PO4 receptor. Similar results have been obtained by Watson et al. (1996), who reported a similar phenomenon when expressing *D. nodosus* *fimA* in *P. aeruginosa* PAK, but not in PAO. A PAK-specific, cell-associated, permissive receptor was suggested to be responsible for this phenomenon. Since the PAK Δ A(pDCA) produces pili that act as receptors for ϕ 6 (data not shown), we suggest that a minor *P. aeruginosa* pilin protein might be serving as the receptor, or as a part of the receptor, for the

Fig. 1. Genetic organization and restriction map of the *Pseudomonas syringae* pv. *tomato* DC3000 *pilA* gene in relation to putative *pilBCD* gene region. Complementation studies and preliminary sequencing show that the genetic organization of *P. syringae* *pilBCD* gene region is similar to that in *P. aeruginosa* as depicted above and resides in the 6.8-kb partially digested *Sau*3AI fragment. Restriction sites used in this study are indicated.

PO4 phage. This minor pilin subunit protein would have to be able to interact with the *P. syringae* PilA in order to be present in the mature pilus composed of the major *P. syringae* pilin subunits. A number of *P. aeruginosa* pilus biogenesis genes have recently been identified that are highly homologous to PilA (Alm et al. 1996; Alm and Mattick 1995, 1996; Russell and Darzins 1994) and may be candidates for a minor pilus component that serves as the PO4 phage receptor in *P. aeruginosa* PAK.

Complementation of *pilB* and *pilC* mutants of *P. aeruginosa*.

As plasmid pDCDA18 had been shown to contain both the *pilA* and *pilD* genes, as well as sequence homologous to the N-terminal coding region of *pilB*, this plasmid was likely to also contain complete copies of *pilB* and *pilC*. To test this possibility, pDCDA18 was introduced into a *pilB* and a *pilC* mutant of *P. aeruginosa* (PAK-B Ω and -C Ω ; Koga et al. 1993), and the resulting transconjugants were tested for PO4

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1   TACGCGCCTTTTCGCTACGTTGGCACGACCTATGCTTCTATCCTTGCAGGGCACTACATA

61  ATTGACGCCTTGGAGAGTAAGAATGAATGCACAAAAGGTTTACTACTGATTGAAGTATGAT
      M N A Q K G F T L I E L M

121 GATCGTAGTTGCGATTGTTGGTATCTTGGCGGCGGTAGCGATTCCGTCATATCAGAACTA
      I V V A I V G I L A A V A I P S Y Q N Y

181 CGCAAAAAAAGCTGCGTATACCGAGGTTCTGGCTGCAATGGCCTCCGTCAAAACTGCTGT
      A K K A A Y T E V L A A M A S V K T A V
      KpnI
241 GGGTGTGTGCGCTGCTCAACAAGGTACCGTTGCTGACTGTGACACTGCAGCTAAAGTAGG
      G V C A A Q Q G T V A D C D T A A K V G

301 TGTAACATTGCCTTCAGGTGCTACTACCGGAGCAGTCAATAAGATGGAAATAACTGCCAC
      V T L P S G A T T G A V N K M E I T A T

361 CAGCGCTGCTATTACTGCTACTCCGAACGCATTTAAAGGTATCCTGACTACCGATACCTG
      S A A I T A T P N A F K G I L T T D T C

421 TAGTCTAACGCCTGCTATAGCAGCTGCTGGATCTCCGGTAACTTGGAGCTACACAGGTGC
      S L T P A I A A A G S P V T W S Y T G A

481 ATGTGTAACCAATGGCTATGTTAAAACTAAGTTTTGAATAT 522
      C V T N G Y V K N

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Fig. 2. Nucleotide sequence of the 522-bp *pilA* encoding fragment from the 1.6-kb *HindIII-XbaI* insert in pSKA16. Predicted PilA amino acid sequence is shown under the sequence of the *pilA* coding region starting at nucleotide 83 and ending at nucleotide 508. Putative consensus RpoN-dependent promoter sequence is underlined. *KpnI* restriction site used for insertion of the Ω fragment in *pilA* and the PilA leader peptide are shown in boldface.

PilA-Ps	M	N	A	Q	K	G	F	T	L	I	E	L	M	I	V	V	A	I	V	G	I	L	A	A	V	A	I	P	S	Y	Q	N	Y	A	K	K	A	A	Y	T	E	V	L	A	A	M	A	S	V	K	T	A	V	G	V	55
TapA-Ah	M	K	K	Q	S	G	F	T	L	I	E	L	M	I	V	V	A	I	V	A	I	L	A	A	I	A	L	P	A	Y	Q	T	Y	T	K	K	A	K	F	T	E	V	V	S	A	T	G	T	Y	K	S	A	L	E	V	55
TfpQ-Mb	M	N	A	Q	K	G	F	T	L	I	E	L	M	I	V	I	A	I	I	G	I	L	A	A	I	A	L	P	A	Y	Q	D	Y	I	S	K	S	Q	T	R	V	V	G	E	L	A	A	G	K	T	A	V	D	A	55	
FimA-Xc	M	K	K	Q	N	G	F	T	L	I	E	L	M	I	V	I	A	I	I	A	I	L	A	A	I	A	L	P	M	Y	Q	D	Y	V	A	K	S	Q	V	T	A	G	L	A	E	I	N	P	G	K	T	Q	Y	E	V	55
PilA-Pa	M	K	A	Q	K	G	F	T	L	I	E	L	M	I	V	V	A	I	I	G	I	L	A	A	I	A	L	P	Q	Y	Q	N	Y	V	A	R	S	E	G	A	S	A	L	A	T	I	N	P	L	K	T	V	E	E	55	
PilA-Ps	C	A	A	Q	Q	G	T	V	A	D	C	D	T	A	A	K	V	G	V	T	L	P	S	G	A	T	T	-	-	G	A	V	N	K	M	E	I	T	A	S	A	A	I	T	A	T	P	N	A	F	K	G	I	L	108	
TapA-Ah	C	F	Q	T	V	G	S	L	L	N	C	-	T	N	G	T	N	G	V	P	P	A	A	G	A	S	G	L	V	T	S	V	A	V	S	G	N	T	A	S	A	A	T	I	T	A	T	G	D	A	T	T	F	G	A	109
TfpQ-Mb	A	L	F	E	G	K	T	P	K	L	G	K	A	A	N	D	T	E	-	-	E	D	I	G	L	T	T	G	G	T	A	R	S	N	L	M	S	S	V	N	I	G	G	A	F	A	T	A	G	T	L	107				
FimA-Xc	A	L	N	E	G	K	T	-	-	-	-	-	-	T	V	A	-	-	D	I	T	E	L	G	L	K	S	P	S	E	R	C	T	I	-	A	P	I	T	A	-	-	-	-	L	S	A	T	G	T	I	93				
PilA-Pa	S	L	S	R	G	I	A	G	S	K	I	K	I	G	T	T	A	S	-	-	T	A	T	E	T	Y	V	G	V	E	P	D	A	N	K	L	-	-	G	V	I	A	V	A	I	E	-	D	S	G	A	G	D	I	104	
PilA-Ps	T	T	D	T	C	S	L	T	P	A	I	A	A	A	G	S	P	V	T	W	S	Y	T	G	A	C	V	T	N	G	Y	V	K	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	142					
TapA-Ah	T	S	N	V	Y	I	M	T	-	-	-	A	A	G	S	N	G	Q	I	I	W	T	A	P	A	S	G	S	T	C	A	G	A	G	F	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	142					
TfpQ-Mb	E	A	T	L	G	-	N	R	A	N	K	D	I	A	G	A	V	I	T	Q	S	R	D	-	A	E	G	V	W	T	C	T	I	N	G	S	A	A	P	G	W	K	S	K	F	V	P	T	G	C	K	E	-	157		
FimA-Xc	E	C	T	L	-	-	K	-	G	N	T	Q	V	V	G	K	K	V	T	L	T	R	A	-	N	D	G	T	W	T	C	K	T	D	A	-	-	-	-	-	L	K	K	Y	A	P	A	G	C	P	G	A	136			
PilA-Pa	T	F	T	F	Q	T	G	T	S	S	P	K	N	A	T	K	V	I	T	L	N	R	T	-	A	D	G	V	W	A	C	K	S	T	Q	-	-	-	-	-	D	P	M	F	T	P	K	G	C	D	N	-	149			

Fig. 3. Sequence alignment of PilA with *Aeromonas hydrophila* TapA, *Moraxella bovis* TfpQ (Marrs et al. 1985), *Xanthmonas campestris* pv. *vesicatoria* FimA and *Pseudomonas aeruginosa* PAO PilA (Sastry et al. 1985). Identical and homologous amino acid residues are boxed.

sensitivity. Phage PO4-sensitive colonies were obtained from both mutants carrying pDCDA18 (data not shown), demonstrating that functional *pilB* and *pilC* homologues were present on this plasmid. Consistent with a previous observation that the *pilMNOP* genes of the two organisms are also very similar in sequence and gene organization (Roine et al. 1996), these results suggest that the gene organization of type IV pilus biogenesis genes in *P. syringae* and *P. aeruginosa* is very similar.

The role of *P. syringae* type IV pili in attachment, UV tolerance, and pathogenesis.

In animal-pathogenic bacteria, type IV pili have been reported to act in adhesion of cells to target tissues (Strom and Lory 1993). In preliminary studies of the role of *P. syringae* ϕ 6-specific pili, they were also proposed to function in attachment to leaf surfaces (Romantschuk and Bamford 1986; Romantschuk et al. 1993; Suoniemi et al. 1995). To test the role of the type IV pili produced by DC3000 in attachment, a *pilA* mutant strain designated DC3000A was constructed by marker exchange (described in Materials and Methods) and, as expected, this strain was resistant to bacteriophage ϕ 6. In attachment studies, however, no reproducible differences could be seen between the *P. syringae* pv. *tomato* DC3000 and the two nonpilated mutant derivatives (E. Roine, unpublished). Similarly, type IV pili of *X. campestris* pv. *vesicatoria* did not have a role in attachment to tomato leaves (Ojanen-Reuhs et al. 1997). Instead, type IV pili were shown to influence aggregation of *X. campestris* pv. *vesicatoria* and tolerance to UV light (Ojanen-Reuhs et al. 1997). Thus, DC3000 wild type, DC3000A, and DC3000A(pDCA) were tested for UV tolerance (Fig. 5). Even after 4 min of exposure the population sizes of the wild type and DC3000A were not significantly different ($P > 0.05$). After 5 min of exposure, however, the DC3000A population size was lower than the detection limit, whereas the population size of the wild type did not drop under the detection limit during the 6-min exposure. The population size of DC3000A(pDCA) was significantly larger than that of DC3000A from 3 min of exposure ($P < 0.05$) until the end of the experiment. These results show that *P. syringae* pv. *tomato* DC3000 wild type was somewhat more tolerant to UV light than the *pilA* mutant strain, and the tolerance was significantly higher if multiple copies of *pilA* were expressed. Further studies are required, however, to determine the exact role of type IV pilus-mediated attachment versus aggregation in various strains of *P. syringae*.

Fig. 4. Complementations of *Pseudomonas aeruginosa pilA* deletion mutant strain PAK Δ A by *P. syringae pilA* gene product cloned in pDN18 (pDCA), which results in sensitivity to both *P. aeruginosa* pilus-specific phage PO4 and *P. syringae* pilus-specific phage ϕ 6. PAK Δ A(pDN18) containing only the plasmid vector remains resistant to both phages.

Pathogenicity of the DC3000 *pilD* and *pilA* mutant strains was tested by infiltrating bacterial suspensions directly into the leaf intercellular spaces of susceptible tomato cultivar Bonny Best. At all inoculation densities, ranging from 10^4 to 10^8 CFU/ml, DC3000A and DC3000D were able to cause bacterial speck symptoms comparable to those produced by the wild type (data not shown). That the *pilD* *P. syringae* mutation had no apparent effect on pathogenicity suggests that the type II protein export pathway is unlikely to play a role in the production of disease symptoms in that strain since, if similar to *P. aeruginosa*, it should also be required for post-translational modifications of components of the type II secretion apparatus.

The role of type IV pili in field fitness.

The leaf surface-associated (epiphytic) population dynamics of the wild-type DC3000 and the DC3000A was tested on a susceptible tomato variant under field conditions. Two experiments were conducted. In the first experiment, the inoculation density of DC3000A on leaves sampled at time zero was significantly lower ($P < 0.005$) than that of the wild type (Fig. 6A). At 24 h after inoculation, after the typical drop in the population size (Wilson and Lindow 1993) the sizes of the two populations were not significantly different from each other ($P > 0.05$). At subsequent sample times, population sizes of DC3000A were consistently lower than those of the wild type, and these differences were statistically significant ($P < 0.05$) on the 10th and 19th day after inoculation. Since the inoculum of DC3000A was significantly smaller, however, it may have influenced the DC3000A population size throughout the experiment (Wilson and Lindow 1994).

In the second experiment, the inoculation densities were identical on leaves sampled at time zero (Fig. 6B). Following the initial drop in population size of both strains, the DC3000A population appeared to rebound significantly faster ($P < 0.05$), but on the 9th day after inoculation until the end of the experiment, the DC3000A population was significantly smaller ($P < 0.05$) than that of the wild type. If *P. syringae*-type IV pili also function in aggregation of bacteria, as was suggested for *X. campestris* type IV pili, the apparent faster

Fig. 5. Survival of *Pseudomonas syringae* pv. *tomato* DC3000 (solid circles), DC3000A (open circles), and DC3000A(pDCA) (solid triangles) after in vitro exposure to UV light. Arithmetic means were calculated from three experiments. Standard errors shown as bars.

rebound of the nonpiliated strain thus existing as single cells can indicate a more even distribution of bacteria over the leaf surface. Consequently, single cells can reach more of the protected sites than the piliated, aggregated wild-type bacteria. In the first experiment, the inoculum density of approximately 5×10^4 of DC3000A, a 1.5 log lower than in experiment two, could be low enough that it did not saturate the protected sites (Wilson and Lindow 1994), and may explain why this faster rebound of DC3000A was not seen in experiment one.

Disease symptoms in both treatments were observed in over half of the sampled leaves in the first and second experiment on the 10th and 5th day after inoculation, respectively, further confirming that the *pilA* gene is not involved in formation of disease symptoms. Although in the first field experiment, the inoculation density of DC3000A on leaves sampled at time zero was significantly lower than that of the wild type, taken together the field results further suggest that pili do play a role

in fitness during foliar colonization and survival. The differences in colonization and survival were smaller than with another strain of *P. syringae* that expresses higher levels of pili also in laboratory conditions (M. Romantschuk, *unpublished*). It may be that in *P. syringae* strains expressing more type IV pili than *P. syringae* pv. *tomato* DC3000, these pili play a bigger role in fitness. Thus, our results further suggest that the role of type IV pili in fitness during colonization and survival on leaf surfaces could be to enhance tolerance to environmental conditions such as exposure to UV light. Further field experiments, however, are required to elucidate the role of type IV pilus in the field fitness of *P. syringae* pv. *tomato* DC3000 and other strains of *P. syringae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Pseudomonas* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) or minimal A salts supplemented with 1% glycerol and 50 mM monosodium glutamate (Davis and Mingioli 1950). In addition, *P. syringae* strains were routinely grown in King's medium B (KB) (King et al. 1954). *E. coli* and *P. aeruginosa* were grown at 37°C, whereas *P. syringae* was grown at 28°C. Antibiotic concentrations (in µg per ml) were as follows: for *P. aeruginosa*, carbenicillin (150), tetracycline (100), kanamycin (50), and streptomycin with spectinomycin (each at 400); for *P. syringae*, carbenicillin (200), tetracycline (15), kanamycin (50), streptomycin with spectinomycin (each at 30), rifampicin (75), and gentamicin (5 to 10); for *E. coli*, ampicillin (100), tetracycline (20), kanamycin (50), and streptomycin with spectinomycin (each at 20).

Genetic techniques.

Total genomic DNA from *P. aeruginosa* was purified as previously described (Strom and Lory 1986). DNA for sequencing was prepared with the Magic Miniprep kit according to manufacturer's instructions (Promega, Madison, WI). Otherwise, standard techniques for subcloning procedures, plasmid preparations, and agarose gel electrophoresis were used (Sambrook et al. 1989). DNA hybridizations were done by the method of Southern (Southern 1975) with Nytran (Schleicher and Schuell, Keene, NH) as support. Labeled probes were prepared by the random-priming method with a nonradioactive kit purchased from Amersham (Arlington Heights, IL). Hybridizations and final washings were done at 45 and 37°C with the protocol described by the manufacturer.

DNA manipulations and sequence analyses.

Broad-host-range plasmids were mobilized into *P. aeruginosa* and *P. syringae* by triparental matings, with pRK2073 or pRK2013 as the mobilizing plasmid. *P. aeruginosa* and *P. syringae* transconjugants were selected on minimal A and King's medium B, respectively, supplemented with the appropriate antibiotics.

To construct a cointegrative plasmid for the generation of DC3000 marker exchange mutants, plasmid pBR322 (Bolivar et al. 1977) was digested with *EcoRI* and *AseI*, the 5' overhanging ends were filled in with the Klenow fragment of DNA polymerase I, and ligated to a *SmaI* gentamicin resistance-

Fig. 6. Epiphytic population sizes of *P. syringae* pv. *tomato* DC3000 (solid circles) and DC3000A (open circles) on leaves of tomato grown in two field experiments. The first experiment (A) was started 5 days before the second (B). Standard errors are shown as bars. Detection limit (solid diamonds) for each sampling point in both experiments was determined as described in Materials and Methods.

encoding cassette from pUC7G (Nunn and Lory 1992). The resultant plasmid was then digested with *EcoRV* and *NruI* and ligated to a *HaeII* fragment containing the multiple cloning site of pBluescript KSII (Stratagene, La Jolla, CA) to give pGSK1.

To introduce the Ω streptomycin/spectinomycin resistance-encoding cassette from pUC19 Ω into the *pilD* gene of *P. syringae*, a *KpnI*-*PstI* fragment containing the *P. syringae pilD* gene was subcloned into pBluescript KSII. A *BglIII*-*BamHI* deletion (*BamHI* site in pBluescript KSII polylinker) was made to delete a *HindIII* site leaving a single unique *HindIII* site within the *pilD* gene. The resulting clone was cut with *HindIII*, the ends were filled with the Klenow fragment, and the Ω cassette was introduced as an *SmaI* fragment. The Ω -containing *pilD* gene was subcloned as an *HindIII*-*XbaI* fragment into the cointegrative plasmid pGSK1 and mobilized into DC3000 in triparental mating with pRK2013 as a helper plasmid, and exconjugants were selected by plating onto KB agar containing rifampicin and gentamicin to select for cointegrates. Resolution of the resultant cointegrates was carried out by passage on antibiotic-free KB broth and screening for gentamicin-sensitive colonies. Southern analyses with *pilD* gene sequences as the probes were used to confirm the correct gene replacement in these resolved cointegrates.

To construct an insertional mutation in the *pilA* gene of DC3000, a 4-kb *SalI* fragment containing the *pilA* gene was cloned into a derivative of pGSK1 in which the *KpnI* site in

the polylinker had been removed. The cloned fragment was cut at the unique *KpnI* site residing in the *pilA* gene, blunt ended with T4 DNA polymerase, and ligated with the *SmaI* fragment containing the Ω cassette. This construct was then mobilized into *P. syringae* DC3000 by triparental mating to isolate gentamicin-resistant cointegrates. Resolution of the resultant cointegrates was carried out by passage on antibiotic-free KB broth and screening for gentamicin-sensitive colonies. The putative gene replacements were confirmed by Southern hybridization.

To delete the *P. aeruginosa pilA* gene, clone pSKSPAK-pilin, containing the *P. aeruginosa pilA* gene on an approximately 2.0-kb *BamHI*-*KpnI* fragment, was used. After *StuI* and partial *ClaI* digestion, the ends were made blunt with Klenow fragment and ligated, resulting in a clone deleted for *pilA* as well as 25 and 32 bp 5' and 3' of the ORF, respectively. This deleted clone was mobilized into PAK wild type and cointegrates were selected on medium containing streptomycin and spectinomycin. One of these cointegrates was selected and incubated with the bacteriophage PO4 (approximately 5×10^7 PFU per plate) on Luria plates without antibiotics. Phage-resistant colonies were selected and the loss of cointegrate was tested by screening for streptomycin/spectinomycin sensitivity. The correct gene replacement was confirmed by Southern hybridization of the total genomic DNA with the original *BamHI*-*KpnI pilA* fragment as the probe.

Table 1. Bacterial strains, phage, and plasmids used in this study

Strain, phage, or plasmid	Relevant traits	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>hsdR</i> , <i>recA</i> , <i>lacZYA</i> , $\phi 80$ <i>dlacZ</i> Δ M15	BRL, Bethesda, MD
<i>Pseudomonas aeruginosa</i>		
PAK	Wild type, Pil ⁺ PO4 ^s	D. Bradley
PAK Δ A	<i>pilA</i> deletion mutant, Pil ⁻ PO4 ^f	This study
PAK-B Ω	<i>pilB</i> mutant with insertion of Ω fragment, Pil ⁻ Sm/Sp ^f PO4 ^f	Koga et al. 1993
PAK-C Ω	<i>pilC</i> mutant with insertion of Ω fragment, Pil ⁻ Sm/Sp ^f PO4 ^f	Koga et al. 1993
PAK-B30	<i>pilD</i> Tn5 insertion mutant, Pil ⁻ Km ^r PO4 ^f	Nunn et al. 1990
<i>P. syringae</i>		
pv. <i>tomato</i> DC3000	Spontaneous Rif ^r derivative of NCPPB1106 (DC52), Pil ⁺ ϕ 6 ^s	D. Cuppels
pv. <i>tomato</i> DC3000D	DC3000 with insertion of Ω fragment in <i>pilD</i> gene, Sm/Sp ^f Pil ⁻ ϕ 6 ^r	This study
pv. <i>tomato</i> DC3000A	DC3000 with insertion of Ω fragment in <i>pilA</i> gene, Sm/Sp ^f Pil ⁻ ϕ 6 ^r	This study
Phage		
PO4	<i>P. aeruginosa</i> pilus-specific phage	ATCC; Bradley 1973
ϕ 6	<i>P. syringae</i> pilus-specific phage	ATCC; Vidaver et al. 1973
Plasmids		
pBluescript-KS/SK II+	Cloning vector, Ap ^r <i>lacZ'</i>	Stratagene, La Jolla, CA
pBR322	Cloning vector, Ap ^r , Tc ^r	Bolivar et al. 1977
pUC19 Ω	2.0-kb <i>SmaI</i> fragment (Ω -fragment) from pHP45 Ω (Prentki and Krisch 1984) cloned into pUC19	S. Lory
pUC7G	Gentamicin resistance-encoding cassette cloned into pUC7 (Vieira and Messing 1982) Ap ^r , Gm ^r	Nunn and Lory 1992
pDN18	Broad-host-range RK2-derived cloning vector with <i>lacZ</i> α and MCS from pTZ18R (Pharmacia), Tc ^r	Nunn et al. 1990
pRK2013	Mobilizing plasmid, Km ^r	Figurski and Helinski 1979
pRK2073	Mobilizing plasmid, Sm ^r	Leong et al. 1982
pGSK1	Suicide vector, Gm ^r	This study
pSKS3	Suicide vector, Sm/Sp ^r	Kagami et al. 1998
pSKSPAK <i>pilA</i>	2.0-kb <i>BamHI</i> - <i>KpnI</i> fragment in pSKS3	D. Nunn
pDCD58	5.8-kb <i>KpnI</i> - <i>PstI</i> <i>P. syringae pilD</i> gene fragment in pDN18	This study
pDCDA18	6.8-kb <i>Sau3AI</i> <i>P. syringae pilA,B,C,D</i> gene fragment in pDN18	This study
pDCDA25	7.1-kb <i>Sau3AI</i> <i>P. syringae pilA,B,C,D</i> gene fragment in pDN18	This study
pDCA	1.6-kb <i>HindIII</i> - <i>XbaI</i> <i>P. syringae pilA</i> gene fragment in pDN18	This study
pKSA16	1.6-kb <i>HindIII</i> - <i>XbaI</i> <i>P. syringae pilA</i> gene fragment in pBluescript KS II	This study
pSKA16	1.6-kb <i>HindIII</i> - <i>XbaI</i> <i>P. syringae pilA</i> gene fragment in pBluescript SK II	This study

DNA sequencing was carried out with the Silver Sequencing System (Promega), according to the manufacturer's directions. DNA fragments for sequencing were obtained from a 1.6-kb *HindIII-XbaI* *P. syringae* *pilA*-containing fragment and subcloned into pBluescript-SKII and KSII. The nucleotide/amino acid sequence data of the *pilA* gene can be found at GenBank under the accession number L35847.

For protein sequence comparisons, the GAP program of the Genetics Computer Group (GCG) program package version 8.2 was used (Devereux et al. 1984). Multiple sequence alignments were generated with programs PILEUP, LINEUP, PRETTY, and PRETTYPLOT of extended GCG (EGCG) version 8.0.

Phage sensitivity assays.

A 20- μ l sample of a suspension (10^{10} PFU/ml) of the pilus-specific phage (PO4, *P. aeruginosa* pilus-specific or ϕ 6, *P. syringae* pilus-specific) was streaked across an L-agar plate, supplemented with appropriate antibiotics. A loopful of the *Pseudomonas* strain to be tested (10^8 CFU/ml) was then streaked across the phage. PO4 plates were incubated either at 37°C (for *P. aeruginosa*) or 28°C (for *P. syringae*), whereas plates with ϕ 6 phage were always incubated at room temperature (21 to 23°C). Resistance was detected as unrestricted growth occurring after passage across the phage streak.

Field experiments.

Field experiments to assess the survival of the *P. syringae* mutants were conducted at the Auburn University EV Smith Research Center in Alabama under permit from the USDA APHIS. Tomato (cv. Agriset 761) plants grown from seed were transplanted into fumigated soil covered by black plastic with drip irrigation in rows approximately 2 m apart. At the time of experimentation tomato plants were approximately 10 to 12 weeks old. Bacterial inocula were grown on KB agar plates supplemented with appropriate antibiotics at 28°C for 2 days. Bacteria were suspended in 10 mM potassium phosphate buffer pH 7.0 to a cell density of approximately 5×10^8 CFU/ml. Three replicate rows of five tomato plants, flanked on all sides by guard (uninoculated) rows, were inoculated with 2 liters of bacterial suspension (approximately 130 ml per plant) with a CO₂-powered back-pack sprayer. Inoculations took place during still weather and a portable plastic "barrier" was used to minimize drift of bacteria. Two consecutive experiments on different plants were conducted at an interval of 5 days. To determine epiphytic bacterial populations, 30 leaflets were collected with alcohol sterilized latex gloves to minimize cross-contamination and placed individually into sterile plastic bags (Fisher Scientific, Pittsburgh, PA). Sterile buffer (20 ml of 10 mM phosphate buffer, pH 7.0) was added to each bag and leaves in the bags were sonicated for 5 min in an ultrasonic bath (model FS28, Fisher Scientific, Pittsburgh, PA) in order to dislodge and suspend bacteria adhering to the leaf surface. Of appropriate dilutions, 50 μ l was plated with a spiral plater (Autoplate 3000, Spiral Biotech, Bethesda, MD) on KB supplemented with appropriate antibiotics and 100 mg of cycloheximide per ml (to suppress fungal growth). Colonies on plates were counted after 60 to 72 h of incubation with a laser counter (Bacterial Colony Counter Model 500A, Spiral Biotech). The mean log-transformed *P. syringae* population size as CFU/cm² estimated from 30 indi-

vidual leaflets was determined for both treatments and each sampling time in both experiments. The detection limit was determined as population density/cm² when the plated sample contained 1 CFU. Population sizes below the detection limit were included as log 0.5 below the detection limit rather than zero (under the assumption that the inoculated bacterium was present on the leaf but was not detectable). Analysis of variance was performed with SPSS for Windows (release 6.1.3; SPSS Inc.).

Pathogenicity tests.

Bacteria to be tested were grown overnight at 28°C with shaking in KB broth supplemented with appropriate antibiotics. Cells were pelleted and resuspended in 10 mM MgCl₂. Cell densities were adjusted to OD_{600nm} of 1.0 corresponding approximately 10^9 CFU/ml, and the lower cell densities were then obtained by serial dilutions. Bacterial suspensions were infiltrated into the lower leaf surface of the tomato cultivar Bonny Best, which is susceptible to DC3000, and plants were incubated in a growth room until symptoms appeared.

Measurement of UV tolerance.

Tolerance to UV light (λ , 254nm) was tested essentially as described by Ojanen-Reuhs et al. (1997) except that aliquots of bacteria were removed at 1-min intervals. Appropriate dilutions were plated on KB. Arithmetic means were calculated from three experiments.

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