The Tat Pathway of the Plant Pathogen *Pseudomonas syringae* is Required for Optimal Virulence

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Pseudomonas syringae is a gram-negative bacterium that infects a number of agriculturally important plant species. The ability of the organism to deliver virulence factors across the plant cell wall is a key to its pathogenicity. Deletion mutants in the twin arginine translocation (Tat) pathway of two pathovars of *P. syringae*, pvs. *tomato* DC3000 and *maculicola* ES4326, displayed a range of pleiotropic phenotypic changes, such as defects in fluorescent siderophore production, a decrease in sodium dodecyl sulfate and copper resistance, and a significant loss in fitness using *Arabidopsis thaliana* or tomato as plant hosts. The genome sequence of *P. syringae* pv. *tomato* DC3000 encodes a number of potential virulence factors that are predicted to be translocated via the Tat pathway, including several proteins involved in iron scavenging (two siderophore receptors, PSPTO3474 and PSPTO3294, and an aminotransferase, PSPTO2155, involved in siderophore biosynthesis). Further candidates for Tat-dependent pathogenicity determinants include the homologs of a cell wall amidase (PSPTO5528), an enzyme involved in periplasmic glucans biosynthesis (PSPTO5542), and two putative phospholipases (PSPTO3648 and PSPTOB0005). Translocation of the putative amidase, aminotransferase, glucans biosynthetic enzyme, and the two phospholipases, but not the two siderophore receptors, is shown to be dependent on the Tat pathway. Strains deleted for the genes encoding the probable aminotransferase and amidase enzymes are significantly less infectious than the wild type. We conclude that the incremental effects due to the failure to correctly localize at least two, and possibly more, Tat substrates gives rise to the attenuated fitness phenotype of the *P. syringae* pv. *tomato* DC3000 *tat* strain.

Additional keywords: iron acquisition, plant pathogen, twin-arginine signal peptide.

Pseudomonas syringae is a gram-negative bacterial plant pathogen taxonomically composed of many different pathovars which individually infect a comparatively narrow number of plant hosts. Collectively, the *P. syringae* pathovars cause dis-

ease on an enormous assortment of plants, inducing a variety of symptoms such as blights, leaf spots, and galls. It is believed to invade plant tissues typically through stomata, colonize the intercellular spaces, and produce necrotic lesions often surrounded by chlorotic halos in host plants. In nonhost or resistant plants, a rapid programmed cell death of plant cells, termed the hypersensitive response (HR), occurs at the site of infection. In this case, the pathogen does not cause disease and is rendered avirulent.

The major virulence determinant of *P. syringae* is the type III secretion system. This delivers >30 effector proteins directly into plant cells via a type III secretion machine encoded by the hypersensitive reaction and pathogenicity (*Hrp*) genes. The structure and mechanism of type III secretion systems has been extensively reviewed (Alfano and Collmer 2004; Collmer et al. 2000, 2002; Jin et al. 2003). The type III apparatus forms a conduit across the two membranes of the bacterium, the plant cell wall and plasma membrane, through which effector proteins are secreted. Once in the plant cell, effectors carry out a number of diverse functions that often are associated with the suppression of plant defense responses (Espinosa and Alfano 2004). In resistant plants, the recognition of one or more effector proteins by a plant resistance (R) protein results in the plant HR that prevents spread of the pathogen and, therefore, attenuation of infection (Dangl and Jones 2001).

In addition to the type III effectors, *P. syringae* secretes a number of other factors that significantly enhance pathogen virulence, including type IV pili, adhesins, siderophores, extracellular polysaccharides, phytotoxins, cell wall-degrading enzymes, and phytohormones (Buell et al. 2003). The flagella is essential for bacterial entry into leaves, and the flagellin protein also is recognized by the plant FLS2 receptor and acts as an elicitor of an HR (Zipfel et al. 2004). *P. syringae* pv. *tomato* DC3000 is a model strain for the species and its genome has been entirely sequenced (Buell et al. 2003). It infects the host plant tomato, causing bacterial speck, but can also infect the model plant *Arabidopsis thaliana*. A closely related strain, *P. syringae* pv. *maculicola* ES4326, infects members of the plant family *Brassicaceae*, including *Arabidopsis* (Dong et al. 1991; Sarkar and Guttman 2004). Hence, although these pathovars are derived from unrelated plants, they both can infect *Arabidopsis*, allowing comparative study of these organisms on identical hosts.

The genome sequence of *P. syringae* pv. *tomato* DC3000, comprising the chromosome (6.4 Mb) and two plasmids, pDC3000A (73.6 kb) and pDC3000B (67.4 kb), encodes 5,763 open reading frames. It encodes genes required for the synthesis of a variety of putative virulence factors and for several different protein delivery pathways, including a type II secretion system (Buell et al. 2003; Preston et al. 2005). As with all

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other bacterial plant pathogens sequenced to date, *P. syringae* pv. *tomato* DC3000 also harbors sequences encoding the *tatABC* genes that are the essential components of the twin-arginine protein translocation (Tat) pathway. The Tat system is a highly unusual protein export pathway that serves to export prefolded proteins across the bacterial cytoplasmic membrane using the energy of the transmembrane proton electrochemical gradient. Substrate proteins (some of which contain redox cofactors) are targeted to the Tat machinery by N-terminal signal peptides that harbor a consensus S/T-R-R-x-F-L-K motif, where the twin arginine residues are almost invariant and are essential for export (Berks 1996; Berks et al. 2000). The Tat system and its substrates have been widely characterized in *Escherichia coli* (Berks et al. 2003). The Tat machinery comprises two types of high molecular weight complexes: a complex comprising stoichiometric amounts of TatB and TatC acts as an initial receptor for substrate proteins (Alami et al. 2003; Bolhuis et al. 2001), while a second complex consisting primarily of TatA is proposed to form the translocation channel (Gohlke et al. 2005). Translocation involves a reversible assembly of TatA onto the substrate-bound TatBC complex that is driven by the protonmotive force (Mori and Cline 2002). Following translocation, substrates either can be anchored at the periplasmic face of the membrane or released into the periplasmic compartment (Hatzixanthis et al. 2003). Although the majority of Tat substrates are periplasmic proteins, at least two Tat-dependent phospholipases are known to be extracellular proteins that are transported across the outer membrane via the type II secretion apparatus (Voulhoux et al. 2001).

Analysis of the Tat system in an increasing number of pathogenic organisms indicates that it makes an important contribution to the virulence of animal and plant pathogens. It has been demonstrated that the *tat* mutants of the animal pathogens *E. coli* (strains K1 and O157) (Crooke et al. 2001; Pradel et al. 2003), *Salmonella typhimurium* (Crooke et al. 2001), *Legionella pneumophila* (De Buck et al. 2004, 2005; Rossier and Cianciotto 2005), and *P. aeruginosa* (Ochsner et al. 2002) and the plant pathogen *Agrobacterium tumefaciens* (Ding and Christie 2003) are significantly attenuated in virulence. The Tat pathway also was shown to be essential for the nitrogen-fixing symbiosis of *Rhizobium leguminosarum* with legumes (Meloni et al. 2003). Analysis of protein secretion in these organisms indicated that the Tat system was variously required for the secretion of phospholipases (Ochsner et al. 2002; Rossier and Cianciotto 2005; Voulhoux et al. 2001), iron-chelating siderophores and uptake receptors (Ochsner et al. 2002), and important bacterial pathogenesis-related processes, including biofilm formation and motility (De Buck et al. 2004, 2005; Ize et al. 2004; Ochsner et al. 2002; Pradel et al. 2003). However, as yet the molecular basis for attenuated virulence of any *tat* mutant strain has not been fully elucidated.

In this work, we show that the Tat pathway is involved in the virulence of two different pathovars of *P. syringae*. Bioinformatic predictions indicate that 30 or so proteins encoded in the *P. syringae* pv. *tomato* DC3000 genome have plausible twin-arginine signal peptides. These include candidate virulence factors such as a cell wall amidase, an enzyme in the pyoverdine biosynthetic cluster, two siderophore receptors, and two phospholipases. We demonstrate that some of these are translocated by the Tat pathway and, by deletion analysis, show that at least two of them contribute to pathogenesis.

RESULTS AND DISCUSSION

Phenotypic analysis of the *P. syringae* *tat* mutant strains.

BLAST analysis of the *P. syringae* pv. *tomato* DC3000 genome sequence using the *E. coli* TatA, TatB, and TatC se-

quences revealed that the *tatABC* genes are present in single copy and that they form a single transcriptional unit, because there is only a 4-bp gap between *tatA* and *tatB*, and a 4-bp overlap between *tatB* and *tatC*. A fourth gene, sharing a 4-bp overlap with *tatC*, encodes a conserved hypothetical protein (PSPTO5158) that probably also forms part of the same transcriptional unit. The four-gene operon is bounded by a gene encoding a P-ribosyl-ATP pyrophosphatase upstream of *tatA* and a gene encoding a methyl-accepting chemotaxis protein downstream of *PSPTO5158*. The organization of the *tat* operon in DC3000 is shown in Figure 1. Unlike the situation with *E. coli* and other enteric bacteria, where a closely related paralog of TatA arose by a relatively late gene duplication event (Sargent et al. 1998; Yen et al. 2002), the *tatA* gene is present in single copy.

The sequence encoding the *P. syringae* pv. *tomato* DC3000 *tatABC* genes was amplified by polymerase chain reaction (PCR) and used to screen a cosmid library of genomic DNA from *P. syringae* pv. *maculicola* ES4326. A 2-kb fragment covering *tatABC* was subcloned and sequenced (GenBank accession number DQ148412). Sequence analysis indicated that the genes have a relatively high degree of identity (<90%) with the DC3000 genes and the organization of the genes and the presence of a fourth gene encoding a homolog of PSPTO5158 is also conserved (Fig. 1).

We constructed an in-frame (nonpolar) deletion of the *tatABC* genes in each of the *P. syringae* pathovars as described below. The *tat* deletion strain of *P. syringae* pv. *tomato* DC3000, ICT1, had an unchanged growth rate when cultured aerobically in either rich or minimal medium with glucose as the only carbon source compared with the parental strain (data not shown). However, ICT1 showed slower growth on iron-depleted medium and, unlike the wild-type strain, did not produce the fluorescent siderophore pyoverdine (Fig. 2A), indicating a role for the Tat pathway in iron scavenging. Such a role also was noted for the Tat pathway in the related animal pathogen *P. aeruginosa* (Ochsner et al. 2002). A recent study has shown that the *tat* mutants of *E. coli* are more sensitive to growth in the presence of copper (Ize et al. 2004). The *P. syringae* pv. *tomato* DC3000 *tat* mutant, ICT1, also was significantly more sensitive to growth in the presence of copper than the cognate wild-type strain (Fig. 2B). The *tat* mutant strains of *P. aeruginosa*, *A. tumefaciens*, and *E. coli* 0157 all exhibited a loss of motility (Ding and Christie 2003; Ochsner et al. 2002; Pradel et al. 2003). However, the motility of the *P. syringae* pv. *tomato* DC3000 *tat* mutant strain was not noticeably impaired (not shown).

The *E. coli* *tat* mutant strains have a defect in cell separation, arising from a mislocalization of two cell wall amidases, that results in the formation of long chains of cells and hypersensitivity to killing by the detergent sodium dodecyl sulfate (SDS) or antibiotics such as ampicillin and erythromycin (Bernhardt and de Boer 2003; Ize et al. 2003; Stanley et al. 2001). Microscopic analysis of the *P. syringae* pv. *tomato* DC3000 *tat* mutant, ICT1, showed that there was no obvious defect in cell separation, and the susceptibility of the strain to growth in the presence of SDS was similar to that of the parental strain (Fig. 2C). Furthermore, sensitivity to antibiotics such as vancomycin also was not affected by the presence of the *tat* mutation (data not shown). These results indicate that there is not an obvious defect in the permeability of the cell envelope in the *P. syringae* pv. *tomato* DC3000 *tat* mutant.

We noted a number of phenotypic differences between *P. syringae* pv. *tomato* DC3000 and pv. *maculicola* ES4326, and also between their cognate *tat* strains. Although the *tat* deletion strain of *P. syringae* pv. *maculicola* ES4326, ICM1, also was not affected for motility and showed an unaltered growth

rate when cultured in rich or minimal medium, it also grew at the same rate as the wild type on iron-depleted medium. Interestingly neither the pv. *maculicola* wild-type nor *tat* mutant strain produced the fluorescent siderophore pyoverdine (not shown), highlighting a significant difference between the two *P. syringae* pathovars. A further difference was noted in the copper sensitivity between the two pathovars—the pv. *maculicola* wild-type strain was more sensitive to the presence of copper than the pv. *tomato* strain (compare Fig. 2B with D) and, interestingly for the *maculicola* strain, the *tat* mutation had no significant effect. It should be noted that differences in copper resistance between *P. syringae* pathovars has been reported previously (Feil et al. 2005). Finally, deletion of the *tat* genes in *P. syringae* pv. *maculicola* ES4326 resulted in a marked sensitivity to growth in the presence of SDS (Fig. 2E). This also was accompanied by a notable increase in sensitivity to antibiotics such as vancomycin (not shown). Taken together, these results indicate that the *tat* mutation has significant, but differing, effects on the physiology of the two *P. syringae* pathovars.

P. syringae *tat* mutant strains are attenuated in virulence.

To assess whether a defect in the Tat pathway resulted in a loss of virulence, growth of the *P. syringae* pvs. *tomato* DC3000 and *maculicola* ES4326 *tat* mutant strains were assessed in planta. Five- to six-week-old *Arabidopsis thaliana* (ecotype

Colombia Col-0) plants were infected with the wild-type and *tat* mutant strains at inocula sizes of 10^5 CFU/ml. After 3 days, leaves were harvested. At this low inoculum size, barely any symptoms of infection could be observed (not shown). However, as shown in Figures 3A and 4A, at this stage of infection, the *tat* mutants of both *P. syringae* pvs. *tomato* DC3000 (Fig. 3A) and *maculicola* ES4326 (Fig. 4A) showed a significantly lower bacterial count than the cognate parental strains (a decrease of 1 to 2 $\log_{10}$). Strains *P. syringae* pv. *tomato* DC3000 and ICT1 ($\Delta tatABC$) also were infiltrated, at CFUs of 10^4 /ml, into leaves of 3 to 4-week-old tomato plants. Again, although symptoms of infection were not obvious 3 days post inoculation, the *tat* mutant strain showed an approximately 1 $\log_{10}$ decrease in growth in planta relative to the wild type (Fig. 3B). Thus, as seen with other bacterial pathogens, the *tat* mutation of *P. syringae* is associated with a significant loss bacterial fitness in planta. The reduced-growth rate of the *P. syringae* pv. *tomato* DC3000 *tat* mutant could be restored by supplying the *tatABC* genes in trans under control of their own promoter (Fig. 3C). Complementation in trans also restored production of pyoverdine and a wild-type level of copper resistance (not shown).

In order to ascertain whether the virulence defect of the *P. syringae* *tat* strains was due to impairment of the type III secretion system, we assessed the ability of the wild-type and *tat* mutants to elicit an HR on plants expressing a cognate *R* gene. A transgenic tomato line expressing *Pto* was infiltrated with a

Fig. 1. Genetic organization of the *tatABC* genes in *Pseudomonas syringae* and *Escherichia coli*. Organization of the *tatABC* genes in **A**, *P. syringae* pv. *maculicola* ES4326 (determined by sequencing the 2-kb insert present in plasmid pKS-PSMtatABC) and **B**, *P. syringae* pv. *tomato* DC3000. Genes *PSPTO5154* and *PSPTO5159* are predicted to encode a P-ribosyl ATP pyrophosphatase and a methyl-accepting chemotaxis protein, respectively. **C**, Organization of the *tatABC* genes in *E. coli* K12. The percent identity between the encoded proteins is marked on the figure.

range of dilutions of the *P. syringae* pv. *tomato* DC3000 wild-type and *tat* mutant strains. The kinetics of HR induction and HR area was identical for both strains (Figure 3D shows the HR after 24 h for inocula size of 5×10^7 CFU/ml). Likewise, *A. thaliana* showed the same HR area and induction time when inoculated with a range of dilutions of the *P. syringae* *maculicola* ES4326 wild-type and *tat* mutant strains, each of which had been transformed with a plasmid constitutively expressing *avrRPM1* (shown 16 h postinoculation from an inoculum of 1×10^8 CFU/ml in Figure 4B). These data indicate that the reduction in fitness observed in *P. syringae* *tat* mutant strains is not the result of an indirect effect on the type III secretion system.

Analysis of Tat-dependent proteins encoded by the genome of *P. syringae* pv. *tomato* DC3000.

In order to identify potential Tat substrates in *P. syringae* pv. *tomato* DC3000 that may be required for optimal virulence of the organism, the genome database was searched using three different Tat signal peptide prediction tools. TatFind (Rose et al. 2002) and TatP (Bendtsen et al. 2005) use complementary methods to search for signal peptide-like sequences that harbor the consensus amino acids of the twin arginine motif, and we applied these algorithms to search the predicted proteins encoded by the pv. *tomato* DC3000 genome. TIGRFAM TIGR01409 is a program devised by TIGR and trained to recognize twin arginine signal peptides that also is complementary to TatFind and TatP, and which TIGR has applied to all of the predicted proteins encoded by the *P. syringae* pv. *tomato* DC3000 genome. Table 1 is a subset of the proteins predicted to contain a putative Tat signal peptide that were recovered by these methods, excluding cytoplasmic proteins, membrane proteins with more than one predicted transmembrane domain (because there is no evidence that these proteins are compatible with export by the bacterial Tat pathway) (Cristóbal et al. 1999; Hatzixanthis et al. 2003), and proteins of unknown function whose bacterial homologs did not contain a twin arginine motif.

Inspection of the proteins listed in Table 1 implicates the Tat system in a number of cellular processes. Two probable copper-binding proteins, CopA (PSPTO3914) and CumA (PSPTO1456), both homologs of multicopper oxidases, have plausible Tat signal sequences, and their mislocalization may account for the increased copper sensitivity of the *P. syringae* pv. *tomato* DC3000 *tat* mutant. In a related organism, *P. putida*, CumA is primarily involved in the oxidation of Mn^{2+} and, therefore, may not contribute significantly to copper resistance (Brouwers et al. 1999). However, *copA*, encoding a periplasmic protein, usually is found in a copper resistance operon comprising four genes, *copA* to *copD* (Cha and Cooksey 1991). Although the DC3000 genome does not encode *copC* and *copD*, it previously has been shown that only *copA* and

copB are essential for copper resistance (Mellano and Cooksey 1988). Interestingly, PSPTO2608, a putative accessory protein predicted to play a role in the delivery of copper to copper enzymes, also is predicted to have a Tat signal peptide.

Fig. 2. Phenotype of *Pseudomonas syringae* *tat* mutant strains. **A**, *P. syringae* pv. *tomato* DC3000 wild-type and the isogenic *tat* mutant strain, ICT1, were grown on solid King's B (KB) medium in the absence (left picture) or presence (right picture) of the iron chelator ethylenediamine(*o*-hydroxyphenylacetic) acid at 50 μ g/ml. Both plates were photographed on a UV transilluminator to detect the production of fluorescent siderophores. Copper sensitivity of wild-type and *tat* mutant strains of **B**, *P. syringae* pv. *tomato* DC3000 (*Pst*) and **D**, *P. syringae* pv. *maculicola* ES4326 (*Psm*). Sodium dodecyl sulfate (SDS) sensitivity of wild type and *tat* mutant strains of **C**, *P. syringae* pv. *tomato* DC3000 and **E**, *P. syringae* pv. *maculicola* ES4326. Copper and SDS sensitivity assays were performed as described by Ize and associates (2004), except that KB medium was used. The error bars represent the standard error of the mean, $n = 2$, except in E, where $n = 3$.

Fig. 3. *Pseudomonas syringae* pv. *tomato* DC3000 *tat* mutant strain is attenuated in virulence. CFU recovered from leaves of **A**, *Arabidopsis thaliana* and **B**, *Lycopersicon esculentum* (tomato) infected with *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and isogenic *tat* (Δtat) mutant strains. Infection tests were performed as described in the text. The error bars represent the standard error of the predicted mean, $n = 6$ (A) and $n = 15$ (B). For each case, the difference in bacterial counts obtained for the wild-type and *tat* strain was statistically significant (A and B, $P = 0.006$ and 0.002 , respectively). **C**, Virulence of the Δtat mutant is completely restored when the *P. syringae* pv. *tomato* DC3000 *tatABC* genes are expressed in trans. Leaves of *A. thaliana* were inoculated with cells of the *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and *tat* (Δtat) strain, and each strain was transformed with plasmid pME-PST*tatABC*. The error bars represent the standard error of the mean, $n = 3$ to 4 . **D**, *P. syringae* pv. *tomato* DC3000 *tat* mutant does not show a reduction in ability to elicit the hypersensitive response (HR) when compared with the wild type. A transgenic *L. esculentum* line expressing *Pto* was infiltrated with cells of the wild-type and *tat* mutant strain as described in the text. Production of the HR was recorded at 24 h post infiltration.

Three proteins listed in Table 1 (PSPTO0985, PSPTO2654, and PSPTO3007) are proposed to bind redox cofactors and may play a role in energy conservation, and a number of putative periplasmic binding protein components of ABC transporters involved in nutrient uptake and other transport processes (PSPTO0249, PSPTO0759, PSPTO1360, PSPTO4078, PSPTO4885, PSPTO5180, and PSPTO5316) contain the canonical twin arginine motif in their signal sequences. Several of the non-cofactor Tat substrates are predicted to be hydrolytic enzymes (PSPTO0005B, PSPTO0491, PSPTO1010, PSPTO2470, PSPTO2806, PSPTO3648, and PSPTO5528).

A number of proteins in Table 1 are reasonable candidates for virulence factors. It is well known that iron acquisition is extremely important in allowing bacterial pathogens to sustain growth in the iron-limited environment of the host. It has been demonstrated in the related pathogen *P. aeruginosa* that the Tat pathway plays a critical role in pyoverdine-mediated acquisition of iron, with up to four putative Tat substrates being involved in this pathway, including an outer membrane receptor (Ochsner et al. 2002). The genome of *P. syringae* pv. *tomato* DC3000 encodes one predicted periplasmic protein that is an

Fig. 4. *Pseudomonas syringae* pv. *maculicola* ES2346 *tat* mutant strain is attenuated in virulence. **A**, CFU recovered from leaves of *Arabidopsis thaliana* infected with *P. syringae* pv. *maculicola* ES2346 wild-type (*Psm*) and isogenic *tat* (Δtat) mutant strains. Infection tests were performed as described in the text. The error bars represent the standard error of the predicted mean, $n = 7$. For each case, the difference in bacterial counts obtained for the wild-type and *tat* strain was statistically significant ($P = 0.002$). **B**, *P. syringae* pv. *maculicola* ES2346 *tat* mutant does not show a reduction in ability to elicit the hypersensitive response (HR) when compared with the wild type. *A. thaliana* Col-0 was infiltrated with the wild-type and isogenic *tat* mutant, each expressing *avrRPM1*, as described in the text. Production of the HR was documented at 16 h post infiltration.

notated as an aminotransferase involved in pyoverdine biosynthesis. The mislocalization of this protein might reasonably account for the absence of fluorescent siderophore production in the *tat* mutant strain. The genome also encodes two putative outer membrane siderophore receptors with twin arginine motifs, one of which was predicted by TatFind to be a Tat substrate and the second of which we found by homology searching. Any or all of these proteins are likely to be involved in pathogen survival in the plant host. Further candidates for Tat-dependent pathogenicity determinants include the DC3000 homolog of MdoD (PSPTO5542) (involved in the biosynthesis of periplasmic glucans and known to play a role in the

virulence of *Erwinia chrysanthemi*) (Page et al. 2001) and the cell wall amidase (PSPTO5528) (a key enzyme in peptidoglycan remodeling and critical for cell envelope integrity in *E. coli*) (Bernhardt and de Boer 2003; Ize et al. 2003). Finally, two putative nonhemolytic phospholipase C proteins, one plasmid (PSPTO0005B) and one chromosomally encoded (PSPTO3648), that share over 90% sequence identity with each other are both predicted to be translocated by the Tat pathway. Phospholipase C proteins hydrolyze phosphatidylcholine present in eukaryotic membranes and, therefore, should facilitate the process of infection. In *P. aeruginosa*, two similar phospholipases, PlcH (hemolytic) and PlcN (nonhemolytic),

Table 1. Compiled prediction of Tat substrates in the *Pseudomonas syringae* pv. *tomato* DC3000 protein database

Substrate	PSPTO no.	Description	TatP	Tatfind	TigrFAMs	Sequence of signal peptide ^a
1	5B	Phosphoesterase family protein (putative phospholipase)	...	x	x	MSDRPEDEDNRNENPS RRRFL GGMAALGAGVTLSGYVSS
2	249	Peptide ABC transporter periplasmic peptide-binding protein	...	x	x	MADNKTDSQLITGQDSLRVFEGLNRLGS RRDAL RMLGVAGVVAAGSTSLFGAA
3	491	Predicted phosphatase, alkaline phosphatase (PhoX)	x	x	x	MKLEENQTTDLENMVGASRAGL TRR GFISAGALCGAAMFLGGNLSRTVLA
4	759	Bmp family protein (lipid binding)	...	...	x	MHLTN PRRP KLLCAMAADVGVIGSSLLASA
5	831	Hypothetical protein (predicted hydrolase)	x	...	...	MGTSMLSFF QRR KTSPTTPSNAAGFTKPESSADA
6	985	Oxidoreductase molybdopterin binding protein	x	x	x	MLIKLPSASGSKESDVTPESIYLS RR TLLASSLAGLAVTALPRWASA
7	991	Hypothetical protein	...	x	...	MLNES RRR AYLTAMQVVNWLPRTLPFAAPSRPELLAPLPPPEVQSIAPASA
8	1010	Alkaline phosphatase D	...	x	x	MTDFNP DRR ITGVAGATLLSILSPFARS
9	1340	Carbonic anhydrase	...	x	x	MAGSGIQTLRQLTNLRLFMNSSL SR RKILQGACCLPVAAALMELPSIANA
10	1360	Bmp family protein (lipid binding)	...	x	x	MSNV DRS FIKLAGIIGLSAALPFGRAYS
11	1456	Multicopper oxidase (CumA)	...	x	x	MS FTRR QILTGIALVAVGLGAGG
12	2141	Cation ABC transporter, periplasmic cation-binding protein	x	...	...	MKEDRLPMPISFK RRP FLRVLLLSLFAA
13	2155	Aminotransferase class V (pyoverdine biosynthesis)	x	x	x	MLAPASFA
14	2470	Senescence marker protein 30-family protein	x	x	x	MT DRRT FLKQAGVLAALPLGSSLATA
15	2608	Sco1/SenC family protein	...	x	x	MDFSPPTTADQ SR RGFLKKSLAVSAT
16	2654	4Fe-4S binding domain protein	...	x	x	VAAIGSLPRLSAA
17	2806	Dienelactone hydrolase family protein	...	x	x	MTYRPT TR REVVTGLSLLSLGLLTGCGNSNA
18	2847	ThiJ/PfpI family protein	x	x	x	MSEPKKHKDAAVDDRAVQ PSRR NFLKFGASGIAAATLSTWIPSDG
19	3007	Flavin amine oxidase	...	...	x	MERLTAKDFAPELLELYDYAHGKINR REF LDRALFSLGGLTAGALLASLS
20	3294	TonB-dependent siderophore receptor putative	...	x	...	MIDNQ RR NLVIGALLAPLAASITLKASA
21	3574	TonB-dependent siderophore receptor putative ^b	...	...	...	MGL TR REALSSIAAVGGEKALKDALAVLGLGPS
22	3598	Dyp-type peroxidase family protein	x	x	x	MRRT LVSLCVIAISQAAWA
23	3648	Acid phosphatase (putative phospholipase)	x	x	x	MSISSQ RR PGPFLLSALSAMFSSGPVHA
24	3914	Copper resistance protein A homolog precursor (CopA)	x	x	x	MKTPDSNPDATNAPVSL QRRR VLMGLGAAGAALASSLSGNVLA
25	4078	Periplasmic amino-acid binding protein	x	...	...	MSDKPEDENTNENP GR RRFLGGMAALGAGVTLSGYVSA
26	4480	Tat pathway signal sequence domain protein	x	x	x	MPT RTSRR TFVKGLAASSILSGLGLWRS
27	4885	Periplasmic amino acid-binding protein	x	x	x	PAWA
28	5180	Cystine ABC transporter, periplasmic cystine binding protein	x	...	...	MDTSLTSTKRS LP RRALGTAALSIIAA
29	5316	Sulfonate ABC transporter, periplasmic sulfonate-binding protein pproteinprotein	...	...	x	SIFGQAAIA
30	5528	N-acetylmuramoyl-L-alanine amidase family 3	...	x	...	MSQFDL KRRR VIQAVGAVGAGLLPGVAPA
31	5542	Glucans biosynthesis protein D precursor (MdoD)	x	x	x	MK RR SLIKAFTLSASIVAMGLTWTVQAMNISAI IR RTFLFSALGLMLGTGIAGQAMAG
						MRTVIL RR SLVALFAAAVALGAVTQAQA
						MHRRQVLLNMLLASAALTLPFGVCA
						MNRRNL LK ASMALAAVGSVSASGLYAARALA

^a List does not include probable nonTat substrates, as described in the text. The putative signal peptide for each protein is given, and the amino acids that match those of the twin arginine motif are highlighted in bold.

^b Found by homology searching.

have been shown to use the Tat system for their translocation across the inner membrane and, subsequently, to be secreted across the outer membrane by the type II secretion pathway (Voulhoux et al. 2001).

Localization of putative Tat-dependent virulence factors.

In order to confirm whether the putative virulence factors identified above are indeed substrates of the *P. syringae* Tat pathway, we examined the localization of epitope-tagged versions of some of these proteins in wild-type and *tat* mutant strains. Western blot analysis of subcellular fractions using an anti-his-tag antibody detects the presence of C-terminally his-tagged MdoD and aminotransferase in the periplasmic fractions of the wild-type strain (Fig. 5A and B). In each case, the periplasmic form of the protein migrated at a slightly lower molecular weight than the protein found in the sphaeroplast fraction, consistent with cleavage of the signal peptide. Both of these proteins were absent from the periplasmic fraction of the *tat* mutant, confirming that they are substrates of the Tat pathway. It should be noted that the Tat dependence of the *E. coli* MdoD protein also has been demonstrated (Lequette et al. 2004).

The work of Ochsner and associates (2002) has implicated the outer membrane ferripyoverdine receptor of *P. aeruginosa*, a predicted β -barrel protein, as a substrate for the Tat pathway in that organism. The twin arginine motif of the signal peptide also is conserved among the signal peptides of two potential siderophore receptors of *P. syringae* pv. *tomato* DC3000 (and also the genomes of some other Pseudomonads); therefore, we sought to test directly whether either of these proteins were substrates for the Tat pathway. A C-terminally his-tagged version of PSPTO3574 (designated Sid1) is found in the outer membrane of both the *P. syringae* pv. *tomato* DC3000 wild-

type and *tat* mutant strain (Fig. 5C). Likewise, although we were not able to express the second potential siderophore receptor (PSPTO3294, designated Sid2) in the native organism, we could express the C-terminally his-tagged protein in *E. coli* (Fig. 5D) where, again, it localized to the outer membrane of both the wild-type and *tat* mutant. Thus, we conclude that, despite having reasonable Tat signal peptides that apparently are conserved among some of the members of this protein family, neither of these putative siderophore receptors are substrates of the *tat* pathway. Indeed, current research suggests that outer membrane β -barrel proteins only attain a folded conformation upon contact with lipopolysaccharide (Bulieris et al. 2003), which is found exclusively outside of the cytoplasm; therefore, it would be unlikely that such proteins would be prefolded before export.

We were not able to detect the predicted amidase or the two putative phospholipases as C-terminal his-tag fusions, indicating that they failed to be properly expressed. Therefore, to test whether the signal peptides of these proteins were capable of engaging with the Tat pathway, we constructed fusions of these sequences to the green fluorescent protein (GFP). GFP has been demonstrated to be a robust reporter for Tat-dependent export in *E. coli* because it is translocated in a folded and, hence, active conformation (Santini et al. 2001; Thomas et al. 2001). A fusion of the signal peptide of the putative amidase to GFP resulted in a distinct halo of periplasmic fluorescence in *P. syringae* pv. *tomato* DC3000 wild-type but not in the *tat* mutant strain (Fig. 6A), consistent with export by the Tat pathway. This result is confirmed in Figure 6B, where fractionation and Western blotting experiments show the mature form of GFP present in the periplasm of the wild-type but not the *tat* strain expressing this fusion. GFP fusions to the two putative phospholipase signal peptides both gave extremely high levels

Fig. 5. *Pseudomonas syringae* pv. *tomato* DC3000 MdoD and aminotransferase homologues are authentic Tat substrates. Analysis of the sphaeroplast (S) and periplasmic (P) fractions prepared from *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and *tat* (Δ *tat*) strains carrying either **A**, pMCS2-*tase* (expressing PSPTO2155_{6his}) or **B**, pMCS2-*mdoD* (expressing PSPTO5542_{6his}). The cytoplasmic (C), total membrane (M), and outer membrane (OM) fractions were prepared from either **C**, *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and *tat* (Δ *tat*) strains, each carrying plasmid pMCS2-*sid1* (expressing PSPTO3474_{6his}) or **D**, *Escherichia coli* strains MC4100 and DADE (As MC4100, Δ *tatABCD*, Δ *tatE*), each carrying pREP4 and pQE-*sid2* (expressing PSPTO3294_{6his}).

of expression, with much of the fusion protein remaining in the cytoplasm, even after extended periods of incubation in the presence of chloramphenicol. However, fractionation of the DC3000 wild-type and *tat* mutant strain expressing these fusions shows the presence of a band corresponding to a processed form of the fusion protein in the periplasm of the wild-type strain only. Therefore, we conclude that two putative phospholipase signal peptides mediate Tat-dependent export of GFP. Collectively, these results confirm that the MdoD homolog, the two putative phospholipases, the putative amidase, and the predicted aminotransferase are substrates of the *P. syringae* Tat pathway.

The Tat-dependent aminotransferase and amidase proteins contribute to *P. syringae* pv. *tomato* DC3000 virulence.

In order to ascertain the underlying basis for the significant loss of fitness in planta of the *P. syringae* pv. *tomato* DC3000 *tat* mutant strain, we examined the effect of deleting genes encoding two of the putative virulence factors identified in silico on the ability of the organism to grow in planta. Given the gen-

eral importance of iron scavenging for pathogenicity, we sought to confirm that the putative aminotransferase was involved in pyoverdine biosynthesis and test whether its mislocalization in the *tat* mutant accounted for the observed reduction in fitness. To this end, we constructed an in-frame deletion of the gene encoding PSPTO2155 as described below. The resultant strain, ICT2, showed the same slow growth on iron-limited medium as the *tat* mutant and also failed to produce the fluorescent siderophore (Fig. 7A). This result strongly suggests that the reason the *tat* mutant does not synthesize pyoverdine is due to its inability to export the putative aminotransferase, and that this protein does, indeed, have a role in siderophore biosynthesis. In planta infections of *Arabidopsis* with ICT2 (designated Δ tase in Fig. 7B) exhibited significantly impaired growth. However, the loss in fitness due to absence of the aminotransferase was not as great as the attenuation seen due to the *tat* mutation. Thus, we conclude that the defect in siderophore production contributes significantly to, but does not account for the all of, the in planta growth defect of the *tat* mutant. Many of the pleiotropic effects of the *E. coli* *tat* mutant can be ascribed to the inability to translocate two Tat-dependent cell wall amidases (Bernhardt and de Boer 2003; Ize et al. 2003, 2004). Therefore, we also sought to test the role of the Tat-dependent amidase protein in the fitness of *P. syringae* pv. *tomato* DC3000. Strain ICT3 (designated Δ ami in Fig. 7B), which carries an in-frame deletion in the gene encoding the putative amidase, also shows a significant loss of fitness compared with the wild-type strain. Again, however, because the decrease in fitness is less than that seen for the *tat* mutant strain, it is clear that the mislocalization of the amidase contributes to, but does not fully account for, the loss of fitness of the *tat* strain. Taken together, the results presented here indicate that the incremental effects due to the failure to correctly localize at least two, and possible more, Tat substrates gives rise to the attenuated in planta growth phenotype of the *P. syringae* pv. *tomato* DC3000 *tat* strain.

Concluding remarks.

We have demonstrated here that, for two different pathovars of the plant pathogen *P. syringae* (pvs. *tomato* DC3000 and *maculicola* ES4326), the *tat* mutation is associated with a significant attenuation in fitness. This adds to a growing number of other pathogens for which the Tat pathway is required for optimal virulence (Crooke et al. 2001; De Buck et al. 2004, 2005; Ding and Christie 2003; Ochsner et al. 2002; Rossier and Cianciotto 2005). To ensure that we maximized identification of the candidate Tat-dependent virulence factors, we combined outputs from three Tat signal peptide prediction methods. Surprisingly, each one of these gave very different results; therefore, we followed this up by manual sorting of the combined list to derive the best estimate for Tat substrate proteins based on our current knowledge of substrate preference for the bacterial Tat pathway. From this approach, we identified six substrates that had the potential to contribute to virulence, inferred from known or probable virulence factors identified in other bacterial pathogens. Interestingly, at least one of these substrates, a predicted outer membrane siderophore receptor, and a closely related homolog, also with a plausible Tat signal peptide, were shown not to be substrates for the Tat pathway. This underscores the importance of verifying substrate predictions because it not only informs on the contribution that the Tat system makes to the physiology of an organism, but it also informs on the types of proteins that are compatible for export by the Tat pathway.

Deletion analysis of genes encoding two Tat substrate proteins has indicated that it is the combined mislocalization of two (or more) proteins that give rise to the considerable loss in fitness of the *P. syringae* *tat* mutant. Because Tat-dependent

Fig. 6. Signal peptides of the *Pseudomonas syringae* pv. *tomato* DC3000 amidase and phospholipase homologues are able to engage the *P. syringae* Tat machinery. **A**, Visualization of halos of periplasmically localized green fluorescent protein (GFP) directed by the signal peptide of the putative amidase (PSPTO5528) in the *P. syringae* pv. *tomato* DC3000 wild-type (left) but not in the isogenic *tat* mutant (right) strain. **B**, Western blot analysis, using anti-GFP antiserum, of sphaeroplast (S) and periplasmic (P) fractions prepared from *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and *tat* (Δ tat) mutant strains, each carrying either plasmid pMCS2-SSAmiC::gfp (top), SSPlcC::gfp (middle), or SSPlcP::gfp (bottom). The positions of the precursor and mature forms of GFP are indicated by the arrows. The asterisk indicates a slightly smaller form of GFP that is found almost exclusively in the cytoplasmic fraction.

amidases and siderophore biosynthetic enzymes are not conserved amongst the pathogens for which the Tat system has been shown to be important for virulence, this suggests that the underlying mechanisms for loss of virulence of bacterial *tat* mutants is likely to differ for each organism. Given the growing interest in exploiting the Tat system as a novel target for antibacterial compounds, an understanding of the underlying basis for loss of virulence of *tat* mutant strains is particularly important for understanding not only the pathogenesis process but also how resistance mechanisms ultimately may arise.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

E. coli strains were cultured aerobically at 37°C in Luria-Bertani (LB) medium with the antibiotics kanamycin (30 µg/ml), tetracycline (10 µg/ml), and ampicillin (200 µg/ml) added where appropriate. *P. syringae* strains were cultured aerobically at 30°C in either King's B (KB) (King et al. 1954) or M9 medium supplemented with 0.4% glucose and 0.04% casamino acids (Sambrook et al. 1989). For growth under low iron conditions, the iron chelator ethylenediamino(*o*-hydroxyphenylacetic) acid was added to KB plates at a concentration of 50 µg/ml. For motility assays, swim plates (KB medium containing 0.25% agar) were inoculated in the center with 5 µl

from an overnight culture and incubated for 48 h at 30°C. Concentrations of antibiotics for *P. syringae* cultures were as follows: rifampicin, 100 µg/ml; tetracycline, 15 µg/ml; and kanamycin, 10 µg/ml.

Copper sensitivity determination in liquid culture was performed as described by Ize and associates (2004), except that strains were cultured in KB medium rather than LB medium. SDS sensitivity assay was performed in liquid cultures as previously described, again in KB medium (Ize et al. 2003). Antibiotic sensitivity experiments were performed by seeding KB top agar with stationary-phase cultures of the strain of interest at 10⁶ CFU/ml. After solidification, 5 µl of a 50-mg/ml vancomycin solution was spotted onto the medium and incubated for 24 h at 30°C. For plant infection tests, overnight cultures of *P. syringae* strains were washed in 10 mM MgCl₂ and resuspended to the appropriate CFU/ml before use.

Strain and plasmid construction.

The bacterial strains and plasmids used in this work are listed in Table 2. In-frame and unmarked deletion mutants in *P. syringae* pv. *tomato* DC3000 were constructed using the suicide vector pLO1 (Lenz et al. 1994). This vector, which contains the conditionally lethal *sacB* gene encoding levansucrase from *Bacillus subtilis*, cannot replicate autonomously in DC3000. However, recombination of the vector onto the chromosome confers kanamycin resistance to the host strain. Subsequently, the loss of

Fig. 7. Tat-dependent putative aminotransferase and amidase proteins contribute to *Pseudomonas syringae* pv. *tomato* DC3000 virulence. **A**, Fluorescent siderophore production by *P. syringae* pv. *tomato* DC3000 is abolished by deletion of the gene encoding the putative aminotransferase. *P. syringae* pv. *tomato* DC3000 wild-type (*Pst*) and *tat* (Δtat), *PSPTO2155* ($\Delta tase$), and *PSPTO5528* (Δami) mutant strains were grown on solid King's B medium in the absence (left picture) or presence (right picture) of the iron chelator ethylenediamino(*o*-hydroxyphenylacetic) acid at 50 µg/ml. Both plates were photographed on a UV transilluminator to detect the production of fluorescent siderophores. **B**, Two Tat substrates, *PSPTO2155* and *PSPTO5528*, contribute to the virulence of *P. syringae* pv. *tomato* DC3000. *Arabidopsis thaliana* infection tests, performed using the same strains as in A, were carried out as described in the text. The error bars represent the standard error of the predicted mean, *n* = 10 to 13. For each case, the difference in bacterial counts obtained for the wild-type and mutant strains was highly statistically significant (*P* < 0.001).

the vector is screened by selecting sucrose-resistant (25% final concentration in solid media) and kanamycin-sensitive recombinant strains, and strains are screened to check for the required genotype by PCR analysis. DNA covering the start codon and approximately 1,000 bp upstream of the gene of interest were amplified by PCR using DC3000 chromosomal DNA as template and cloned into pLO1. Next, DNA covering the last few codons of the gene of interest and approximately 1,000 bp downstream was amplified by PCR and cloned into pLO1 already carrying the upstream region. Oligonucleotide sequences used to amplify the fragments are available on request. The pLO1-based deletion alleles were mobilized onto the DC3000 wild-type chromosome by single crossover using *E. coli* S17-1 as the delivery strain, and counter-selecting for *P. syringae* by plating out the mating mixture onto solid KB medium containing rifampicin (150 µg/ml final concentration) as well as kanamycin. Double crossover events subsequently were selected by growth in the presence of sucrose as described above.

The *tatABC* mutant of *P. syringae* pv. *maculicola* ES4326 was generated using the PCR-targeting approach of Datsenko and Wanner (2000). The kanamycin resistance gene (encoded on plasmid pKD4) was amplified with primers designed to prime either at the start or end of the gene, with each primer also incorporating 39 nucleotides identical to the start of *tata* or end of *tatC*. *E. coli* strain BW25113 harboring the plasmids pKD20 and pCC1 was electroporated with the PCR-

amplified kanamycin gene, and double crossovers were selected by plating the transformants onto LB plates containing kanamycin. Plasmid pCC1 carrying the $\Delta tatABC::Kan^R$ allele was isolated by maxiprep and transformed by electroporation into *P. syringae* pv. *maculicola* ES4326. Because pLAFR3 can replicate in *P. syringae*, serial subcultures of the transformants were grown without tetracycline. After five rounds of subculture, kanamycin-resistant, tetracycline-sensitive colonies were identified and shown to carry the $\Delta tatABC::Kan^R$ allele by PCR analysis.

The *tat* operon of *P. syringae* pv. *tomato* DC3000 comprising the *tatABC* genes, together with 1,000 bp of DNA upstream of the *tata* start codon, were amplified by PCR. The product was digested with *Xho*I and *Eco*RI and cloned into the vector pME6030, that previously had been digested with the same enzymes, to produce plasmid pME-PST*tatABC*. Plasmid pJF1 was constructed by amplification of the *avrRPM1* gene, digestion of the PCR product, and cloning into the broad host range vector pME6012. Expression of *avrRPM1* from this construct is under control of the constitutive kanamycin promoter. Plasmid pME-*sid1* carries the gene encoding PSPTO3474 in frame with a C-terminal hexa-histidine tagging sequence in the broad host range vector pME6030. It was constructed following PCR amplification of the gene (lacking its stop codon), digestion with *Nco*I and *Bam*HI restriction enzymes, and cloning into pQE60 that had been pre-

Table 2. Strains and plasmids used in this study

Strain	Relevant genotype ^a	Source or reference
<i>Escherichia coli</i>		
DH5α	supE44ΔlacU169 (Ψ80lacZΔM15) hsdR1 recA1 gyrA96 thi-1 relA1	BRL, Gaithersburg MD, U.S.A.
S17-1	Sm ^r Sp ^r hdsR (RP4-2 kan::Tn7 tet::Mu integrated in the chromosome)	Simon et al 1983
BW25113	K12 derivative: ΔaraBAD, ΔrhaBAD	Datsenko and Wanner 2000
XL1 blue MR	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac	Stratagene
MC4100	F ⁻ ΔlacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301	Casadaban and Cohen 1979
DADE	As MC4100, ΔtatABCD ΔtatE	Wexler et al 2000
<i>Pseudomonas syringae</i>		
<i>P. syringae</i> pv. <i>maculicola</i> ES4326	Wild type, Rif ^r	Davis et al 1991
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Wild type, Rif ^r	Cuppels 1986
ICM1	As <i>P. syringae</i> pv. <i>maculicola</i> ES4326, ΔtatABC::Km ^r	This work
ICT1	As <i>P. syringae</i> pv. <i>tomato</i> DC3000, ΔtatABC	This work
ICT2	As <i>P. syringae</i> pv. <i>tomato</i> DC3000 ΔPSPTO2155	This work
ICT3	As <i>P. syringae</i> pv. <i>tomato</i> DC3000, ΔPSPTO5528	This work
Plasmids		
pLAFR3	Tet ^r IncP1 cos ⁺ rlx ⁺	Staskawicz et al 1987
pKD20	Amp ^r λ-RED (<i>gam</i> , <i>bet</i> , <i>exo</i>), <i>bla</i> , <i>araC</i> , <i>rep101</i> ^{ts}	Datsenko and Wanner 2000
pKD4	Km ^r <i>bla</i> FRT <i>ahp</i> FRT PS1 PS2 oriR6K	Datsenko and Wanner 2000
pBluescript (II KS ⁺)	Amp ^r	Stratagene
pLO1	<i>sacB</i> RP4 <i>oriT</i> Cole1 <i>ori</i> Km ^r	Lenz et al 1994
pBBR1MCS-2	Km ^r	Kovach et al 1995
pME6030	Tet ^r	Heeb et al 2000
pME6012	Tet ^r	Heeb et al 2000
pQE60, pQE70	C-terminal His-tag expression vectors	Qiagen
pREP4	Km ^r <i>lacI</i>	Zamenhof and Villarejo 1972
pRR-GFP	pBAD24, ssTorA::GFP	Santini et al 2001
pCC1	pLAFR3 carrying <i>P. syringae</i> pv. <i>maculicola</i> ES4326 <i>tatABC</i>	This work
pKS-PSM <i>tatABC</i>	pBluescript containing 2-kb fragment carrying <i>tatABC</i> from <i>P. syringae</i> pv. <i>maculicola</i> ES4326	This work
pJF1	As pME6012, <i>avrRPM1</i>	This work
pME-PST <i>tatABC</i>	pME6030, containing <i>tatABC</i> and promoter region from <i>P. syringae</i> pv. <i>tomato</i> DC3000	This work
pME- <i>sid1</i>	As pME6030, PSPTO3474 _{6his} ⁺	This work
pQE- <i>sid2</i>	As pQE70, PSPTO3294 _{6his} ⁺	This work
pMCS2- <i>tase</i>	As pBBR1MCS-2, PSPTO2155 _{6his} ⁺	This work
pMCS2- <i>mdoD</i>	As pBBR1MCS-2, PSPTO5542 _{6his} ⁺	This work
pMCS2- <i>SSami::gfp</i>	As pBBR1MCS-2, ssPSPTO5528::GFP	This work
pMCS2- <i>SSplcC::gfp</i>	As pBBR1MCS-2, ssPSPTO3648::GFP	This work
pMCS2- <i>SSplcP::gfp</i>	As pBBR1MCS-2, ssPSPTO5B::GFP	This work

^a Sm^r, Sp^r, Rif^r, Tet^r, Amp^r, and Km^r = streptomycin-, spectinomycin-, rifampicin-, tetracycline-, ampicillin-, and kanamycin-resistant, respectively. GFP = green fluorescent protein.

digested with the same enzymes. The *PSPTO3474_{his}* allele along with the phage T5 promoter present on pQE60 subsequently were excised by digestion with *XhoI* and *HindIII* and cloned into pME6030 that had been predigested with the same enzymes. The genes encoding PSPTO3294, PSPTO2155, and PSPTO5542 all were cloned in an analogous manner, initially by PCR amplification of each gene minus its stop codon, digestion with *BamHI* and *SphI*, and ligation into pQE70 that had been digested with the same enzymes. Subsequently, the C-terminally his-tagged alleles of each gene along with the phage T5 promoter present on pQE70 were cloned following digestion with *XhoI-HindIII* into the broad host range vector pBBR1MCS-2 that had been predigested with the same enzymes. Plasmids carrying GFP fusions to the signal sequences of PSPTO5B, PSPTO3648, and PSPTO5528 all were constructed in an analogous manner. First, the *gfp-mut2* gene present on pRR-GFP was amplified by PCR and cloned into pBBR1MCS-2 by digestion with *BamHI* and *XbaI*. DNA covering the signal peptide coding regions of PSPTO5B, PSPTO3648, and PSPTO5528 as far as two amino acids downstream of the predicted cleavage sites (Table 1) was amplified by PCR and cloned into pQE70 by digestion with *SphI* and *BamHI*. Subsequently, the signal peptide coding regions for each sequence, along with the phage T5 promoter present on pQE70, were cloned following digestion with *XhoI* and *BamHI* into pBBR1MCS-2 containing the *gfp-mut2* gene.

All constructs were verified by DNA sequencing (John Innes Genome Laboratory, John Innes Centre, U.K.) and subsequently were transferred to *P. syringae* strains by electroporation. Primer sequences for all constructs are available on request.

Construction and screening of a cosmid library of *P. syringae* pv. *maculicola* ES4326.

A cosmid library was prepared in pLAFR3 from *P. syringae* pv. *maculicola* ES4326 chromosomal DNA as described (Staskawicz et al. 1987), packaged into lambda phage heads using the Gigapack III packaging system, and transduced in *E. coli* XL1-blue MR, all according to the supplier's instructions (Stratagene, La Jolla, CA, U.S.A.). Colony lifts were prepared from the plated library using Hybond N+ filter disks according to the supplier's instructions (Amersham Biosciences UK Limited, Bucks, U.K.). The cosmid library was screened using a heterologous probe containing the *P. syringae* pv. *tomato* DC3000 *tatABC* genes which was generated by PCR from DC3000 genomic DNA using the primers IC1 (5'-AAGCCCCA TGTTCGGTATCAG-3') and IC2 (5'-TACTTGACGTCGACA ATGCC-3'). The fragment was radiolabeled using ³²P-CTP and the Ready-To-Go DNA labeling kit according to the manufacturer's instructions (Amersham Biosciences). Filter disks were washed at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Sambrook et al. 1989) and 0.1% SDS and exposed to Fuji Medical X-Ray film. A single cosmid, pCC1, was identified that reacted strongly with the probe. Cosmid DNA was isolated and digested with *HindIII*, and the fragments were analyzed by Southern blot (Sambrook et al. 1989) using Church buffer (0.25 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 1% bovine serum albumen, and 7% SDS) for hybridization, and the same probe used for library screening. Washes were performed at 65°C in 2× SSC and 0.1% SDS. A 2-kb *HindIII* fragment containing the entire *tat* operon was identified, and this subsequently was subcloned into pBluescript II KS+ and the DNA sequence determined.

Protein methods.

For expression of fusion proteins in *P. syringae* strains, cultures were grown normally at 30°C until they reached an optical density at 600 nm (OD₆₀₀) of approximately 0.6, after

which time cells were harvested and fractionated as described below. *P. syringae* strains carrying plasmids pMCS2-*SSplcC::gfp* or pMCS2-*plcP::gfp* were cultured at 16°C and protein synthesis was blocked by the addition of chloramphenicol at 10 µg/ml final concentration for 120 min before fractionation. For expression of fusion proteins in *E. coli* harboring pREP4, strains were cultured at 37°C. Once an OD₆₀₀ of 0.6 was attained, production of the recombinant protein was induced by the addition of 1 mM IPTG and cells were harvested after a further 90 min.

Periplasmic and spheroplast fractions of *E. coli* and *P. syringae* both were prepared according to the lysozyme/EDTA method, and membrane fractions subsequently were prepared from sphaeroplasts by cold osmotic shock (Osborn et al. 1972). Total membranes were isolated as described previously (De Leeuw et al. 2001). For preparation of outer membranes, total membranes were resuspended in 50 mM triethanolamine and 250 mM sucrose. Sarkosyl (0.5% final concentration), which selectively solubilizes the inner membrane, was added to the suspension, which then was incubated at room temperature under stirring for 20 min. Outer membranes were collected by ultracentrifugation (Nikaido 1994). SDS polyacrylamide gel electrophoresis was carried out according to Laemmli (Laemmli 1970). Immunoblotting was performed using the ECL method (Amersham Biosciences) according to the manufacturer's instructions. Anti-pentahistidine tag antibody was obtained from Qiagen. Anti-GFP was purchased from Roche. Protein concentrations were estimated according to the method of Lowry and associates (1951).

Microscopy.

For fluorescent microscopy, *P. syringae* strains expressing GFP fused to the amidase signal sequence were cultured at 30°C until mid-exponential phase, after which protein synthesis was blocked by the addition of chloramphenicol at 10 µg/ml final concentration for 50 min before fixation. After washing, the cells were fixed in a low melting point agarose-NaCl solution and samples were examined by phase-contrast and fluorescent microscopy. For deconvolution analysis, a series of z-axis sections was taken at 0.5-µm intervals with an exposure time of 500 ms per section. The images were deconvoluted using the software AutoDeblur (AutoQuant Imaging, Inc., Troy, NY, U.S.A.). The preparation of the samples for electron microscopy procedure has been described elsewhere (Stanley et al. 2001).

Plant assays.

For pathogenesis experiments, 5 to 6-week-old *A. thaliana* ecotype Columbia Col-0 plants were infected by infiltrating with a bacterial suspension at 10⁵ CFU/ml in 10 mM MgCl₂ into plant leaves using a needleless syringe. Three- to four-week-old tomato plants (*Lycopersicon esculentum*) were infiltrated by applying vacuum pressure for 2 min. The surfactant L-77 Silwet (Lehle Seeds, Round Rock, TX, U.S.A.) used at 0.02% final concentration was added to the bacterial inoculum which had been adjusted to 10⁴ CFU/ml. Bacterial levels in planta were determined after 3 days of infection by cutting leaf disks with a boring tool (inner diameter 0.7 cm) and placing the plant material in 1 ml of KB medium. The disks were homogenized completely and the resulting suspension, containing the bacteria, was diluted and plated on KB plates containing rifampicin (50 µg ml⁻¹). To perform HR assays, leaves from resistant tomato lines (Rio Grande 76R) (Chang et al. 2000) or *A. thaliana* ecotype Columbia Col-0 were infiltrated with a range of bacterial inocula from 10⁶ to 10⁸ CFU/ml using a needleless syringe. Development of symptoms was observed between 15 and 24 h after infiltration. For statistical analysis,

the generalized linear model (analysis of variance) was applied using the Genstat for Windows (8.1 edition; VSN International, Oxford, U.K.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Pseudomonas syringae* Genome Resources home page:
www.pseudomonas-syringae.org
 TIGR (The Institute for Genomic Research) TIGRFAMs webpage:
www.tigr.org/TIGRFAMs