Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2012) 13(7), 764–774 DOI: 10.1111/J.1364-3703.2012.00789.X

Type IV pilin is glycosylated in *Pseudomonas syringae* pv. *tabaci* 6605 and is required for surface motility and virulence

LINH CHI NGUYEN[1], FUMIKO TAGUCHI[1], QUANG MINH TRAN[1], KANA NAITO[1], MASANOBU YAMAMOTO[2], MAYUMI OHNISHI-KAMEYAMA[2], HIROSHI ONO[2], MITSURU YOSHIDA[2], KAZUHIRO CHIKU[2], TADASHI ISHII[2], YOSHISHIGE INAGAKI[1], KAZUHIRO TOYODA[1], TOMONORI SHIRAISHI[1] AND YUKI ICHINOSE[1,*]

[1]*Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka 1-1-1, Kita-ku, Okayama 700-8530, Japan*
[2]*National Food Research Institute, NARO, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*

SUMMARY

Type IV pilin (PilA) is a major constituent of pilus and is required for bacterial biofilm formation, surface motility and virulence. It is known that mature PilA is produced by cleavage of the short leader sequence of the pilin precursor, followed by methylation of N-terminal phenylalanine. The molecular mass of the PilA mature protein from the tobacco bacterial pathogen *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta* 6605) has been predicted to be 12 329 Da from its deduced amino acid sequence. Previously, we have detected PilA as an approximately 13-kDa protein by immunoblot analysis with anti-PilA-specific antibody. In addition, we found the putative oligosaccharide-transferase gene *tfpO* downstream of *pilA*. These findings suggest that PilA in *Pta* 6605 is glycosylated. The defective mutant of *tfpO* (Δ*tfpO*) shows reductions in pilin molecular mass, surface motility and virulence towards host tobacco plants. Thus, pilin glycan plays important roles in bacterial motility and virulence. The genetic region around *pilA* was compared among *P. syringae* pathovars. The *tfpO* gene exists in some strains of pathovars *tabaci*, *syringae*, *lachrymans*, *mori*, *actinidiae*, *maculicola* and *P. savastanoi* pv. *savastanoi*. However, some strains of pathovars *tabaci*, *syringae*, *glycinea*, *tomato, aesculi* and *oryzae* do not possess *tfpO*, and the existence of *tfpO* is independent of the classification of pathovars/strains in *P. syringae*. Interestingly, the PilA amino acid sequences in *tfpO*-possessing strains show higher homology with each other than with *tfpO*-nonpossessing strains. These results suggest that *tfpO* and *pilA* might co-evolve in certain specific bacterial strains.

INTRODUCTION

Type IV pili (T4P) are filamentous polymers of pilin protein subunits (PilA) that extend from many Gram-negative bacterial surfaces (reviewed in Mattick, 2002; Nudleman and Kaiser, 2004; Strom and Lory, 1993). These pili contribute to various bacterial behaviours, including adhesion to biotic and abiotic surfaces, surface twitching and swarming motilities, biofilm formation and bacteriophage adsorption. Thus, they are thought to be involved in bacterial pathogenicity by initiating the colonization on host tissue through adhesion to and motility across the surface (Burdman *et al*., 2011; Mattick, 2002). In phytopathogenic bacteria, such as *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta* 6605), *Acidovorax avenae* ssp. *citrulli* and *P. syringae* pv. *tomato* DC3000, T4P promote virulence towards their respective host plants (Bahar *et al*., 2009; Burdman *et al*., 2011; Roine *et al*., 1998; Taguchi and Ichinose, 2011). Furthermore, T4P in *Pta* 6605 are known to be involved in the hypersensitive reaction-inducing activity in nonhost *Arabidopsis thaliana* leaves, as the *pilA*-defective mutant (Δ*pilA*) of this pathogen shows a remarkably reduced expression of *hypersensitive reaction and pathogenicity* (*hrp*)-related genes (Taguchi and Ichinose, 2011).

Recently, protein glycosylation has been observed not only in eukaryotes, but also in prokaryotes, in both Gram-negative and Gram-positive bacteria (Benz and Schmidt, 2002; Ichinose *et al*., 2011; Logan, 2006; Power and Jennings, 2003). Glycans are often used to decorate proteins at the bacterial surface or components of appendages, such as flagella and pili. Pilin glycosylation has been reported in a modest number of animal pathogenic bacteria. However, pilin glycosylation is not species specific, but strain specific. Among the five distinct phylogenetic groups (I–V) based on the PilA amino acid sequence and the presence of specific accessory genes in *P. aeruginosa* (*Pa*), pilins of bacteria from groups I (e.g. *Pa* 1244) and IV (e.g. *Pa* 5196) were found to be glycosylated (Castric, 1995; Kus *et al*., 2004, 2008). Similar strain-specific pilin glycosylation was also found in *Neisseria meningitidis* and *N. gonorrhoeae* (Aas *et al*., 2007; Virji *et al*., 1993). Pilins of both *N. meningitidis* and *N. gonorrhoeae* are glycosylated at serine 63 (Ser63) by the attachment of a short oligosaccharide of up to three sugar residues in length by the glycosyltransferases PglL and PglO, respectively (Parge *et al*., 1995; Power *et al*., 2006; Stimson *et al*., 1995). In the case of *P. aeruginosa*, *Pa* 1244 requires TfpO (originally called PilO) in order to transfer a single O-antigen repeating unit of lipopolysaccharide (LPS) to the C-terminal Ser residue of PilA (Castric, 1995; Castric *et al*., 2001; Comer *et al*., 2002), and *Pa* 5196 pilins are modified at multiple sites with trisaccharides of

**Correspondence*: Email: yuki@cc.okayama-u.ac.jp

α-1,5-D-Ara*f* by arabinosyltransferase TfpW (Kus *et al*., 2008; Voisin *et al*., 2007). Both *tfpO* in *Pa* 1244 and *tfpW* in *Pa* 5196, encoding pilin glycosyltransferases TfpO and TfpW, respectively, localize immediately downstream of *pilA*. The *Pa* 1244 Δ*tfpO* mutants produced functional and nonglycosylated pili with reduced twitching motility, increased surface hydrophobicity and less virulence relative to the wild-type (WT) strain (Smedley *et al*., 2005). Similarly, the defective mutant strains for *tfpW* and the glycan biosynthesis-related genes in *Pa* 5196 showed reductions in motility, molecular mass of the pilin protein and pilus assembly relative to the WT strain (Harvey *et al*., 2011; Kus *et al*., 2008). These reports suggest that the glycan may contribute to T4P adhesiveness, thereby enhancing colonization and virulence.

Pseudomonas syringae, an opportunistic plant pathogenic bacterial species, can be classified into pathovars according to their host ranges and disease symptoms. *Pta* 6605 is a bacterium causing wildfire disease in tobacco plants (Ichinose *et al*., 2003). Previously, another filamentous fibre, flagellin of *Pta* 6605, was revealed to be glycosylated by three glycosyltransferases: Fgt1, Fgt2 and VioT (Ichinose *et al*., 2011; Nguyen *et al*., 2009; Taguchi *et al*., 2006). The *fgt1* and *fgt2* genes are located just upstream of the flagellin gene *fliC*, whereas *vioT* is located in the gene cluster to synthesize the substrate of VioT, dTDP-*N*-(3-hydroxy-1-oxobutyl)-2-*O*-methylviosamine. Glycosylation of the flagellin protein was found to be essential for surface motility, the stability of flagellar filaments and total virulence (Ichinose *et al*., 2011; Taguchi *et al*., 2008, 2009). Recently, we have found that this bacterial strain possesses T4P composed of major PilA subunits and several minor pilin subunits (Taguchi and Ichinose, 2011). Notably, the Δ*pilA* mutant exhibits the absence of T4P, followed by the loss of surface swarming motility and reduced biofilm formation and virulence. This indicates that T4P are required for virulence in this pathogen. The amino acid sequence of PilA in *Pta* 6605 was deduced from the *pilA* nucleotide sequence (Taguchi and Ichinose, 2011). Interestingly, the C-terminal residue of PilA in *Pta* 6605 was Ser, similar to that of *Pa* 1244 pilin. It is known that the C-terminal Ser residue is a unique glycosylation site in *Pa* 1244 pilin (Comer *et al*., 2002). However, pilin glycosylation has not been reported in any phytopathogenic bacteria, including *P. syringae*. In the present study, we found a putative pilin glycosyltransferase gene, *tfpO*, immediately downstream of *pilA* in *Pta* 6605. We report here on pilin glycosylation in *Pta* 6605 and the roles of pilin glycan in surface swarming motility and virulence.

RESULTS

tfpO gene in *Pta* 6605

Based on the genomic information at the *Pseudomonas* Genome Database V2 website (http://v2.pseudomonas.com), we attempted to isolate a DNA fragment containing *pilA* and its downstream region in *Pta* 6605. A pair of polymerase chain reaction (PCR) primers (PilA-F and PilA-R) was designed based on the registered sequence (accession number CP000058) of *pilB* (PSPPH0820) and the *open reading frame 1* (*orf1*, PSPPH0825) in *P. syringae* pv. *phaseolicola* (*Pph*) 1448A. A 5533-bp DNA fragment including *pilA* was amplified by PCR and the sequence was analysed. There are two *open reading frames* (*orf*) downstream of *pilA* (Fig. 1). The putative function and amino acid identity of each protein product are shown in Table 1. The first ORF (immediately downstream of *pilA*) has 86% amino acid identity to a putative glycosyltransferase of *P. savastanoi* pv. *savastanoi* 3335 (*Psav* 3335) and 37% identity to the pilin glycosyltransferase TfpO of *Pa* 1244 (Castric, 1995). Therefore, we refer to this gene as *tfpO* hereafter. Furthermore, TfpO in *Pta* 6605 has 19% identity over 225 residues and 17% identity over 388 residues with oligosaccharyltransferases TfpW of *Pa* 5196 (Kus *et al*., 2004) and PglL of *N. meningitidis* (Power *et al*., 2006), both glycosylates of the respective pilin proteins. These findings led us to hypothesize that this gene is involved in pilin glycosylation. The second ORF showed significant homology with the gene encoding the polysaccharide biosynthesis protein in *Xanthomonas axonopodis* pv. *citri* strain 306, with 41% amino acid identity, and *Acidovorax avenae* ssp. *avenae* ATCC 19860, with 34% identity, and was named *psb*.

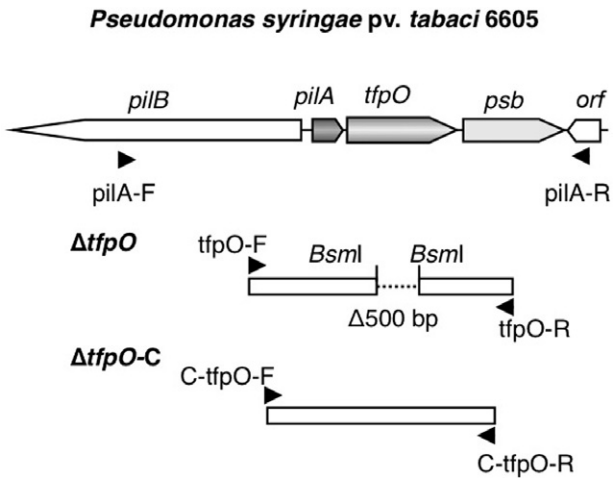

Fig. 1 Schematic organization of *pilA* and its surrounding region in *Pseudomonas syringae* pv. *tabaci* 6605. The construction of the *tfpO* mutant and its complemented strain with a DNA fragment containing *pilA* and *tfpO* is shown. Sets of polymerase chain reaction (PCR) primers were used for PCR, as indicated by the arrows. To generate the internal deletion, digestion of the PCR product by primers (tfpO-F and tfpO-R) was performed with *Bsm*I for Δ*tfpO*. For complementation of the Δ*tfpO* mutant, a DNA fragment containing *pilA*, *tfpO* and a putative promoter sequence was amplified with C-tfpO-F and C-tfpO-R.

Influence of *tfpO* mutation on pilin structure

The amino acid sequence of PilA *Pta* 6605 has been reported previously (Taguchi and Ichinose, 2011). According to Strom

Table 1 Identities and putative functions of open reading frames (ORFs) in the *pilA* region of *Pseudomonas syringae* pv. *tabaci 6605*.

ORF	Size (amino acids)	Putative function of homologues	Organisms	NCBI protein accession number	Amino acids (%)
pilA	131	Type IV pilin	*Psav* 3335	ZP_07003592.1	97
			Pa 1244	CAE18458	28
			Pa 5196	AAM52059	29
tfpO	476	Glycosyltransferase	*Psav* 3335	ZP_07003593	86
			Pa 1244	CAA58769	37
			Pa 5196	AAM52060	19
			Nm MC58	AAF41024	17
Psb	428	Polysaccharide biosynthesis protein	*Xac* 306	NP_640412.1	41
			Aaa	YP_004233424.1	34

Abbreviations for organisms: *Aaa*, *Acidovorax avenae* ssp. *avenae* ATCC19860; *Nm*, *Neisseria meningitidis*; *Pa*, *Pseudomonas aeruginosa*; *Psav*, *Pseudomonas savastanoi* pv. *savastanoi*; *Xac*, *Xanthomonas axonopodis* pv. *citri*. NCBI, National Center for Biotechnology Information.

et al. (1993), a short leader sequence of a pilin precursor was cleaved, and the mature PilA was produced by the methylation of N-terminal phenylalanine by a bifunctional PilD protein with prepilin peptidase and *N*-methyltransferase. The molecular mass of the PilA mature protein was predicted to be 12 329 Da. We detected PilA as an approximately 13-kDa protein in the whole-cell lysate of *Pta* 6605 by immunoblot analysis with anti-PilA-specific antibody (Fig. 2A). A glycostaining experiment of partially purified pilin protein from the *Pta* 6605 WT strain showed a positive signal, indicating that PilA is glycosylated (Fig. 2B).

To examine the potential role of TfpO in pilin glycosylation, the *tfpO*-defective mutant and its complemented strain in *Pta* 6605 were generated, and designated as Δ*tfpO* and Δ*tfpO*-C, respectively. As expected, the Δ*tfpO* mutant pilin migrated more rapidly than that of the WT strain on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), whereas that of the Δ*tfpO*-C strain showed similar mobility to the WT pilin (Fig. 2A). In addition, the glycostaining experiment indicated that PilA prepared from the Δ*tfpO* mutant was not stained as a glycoprotein, whereas a positive band was again detected in PilA of the Δ*tfpO*-C strain (Fig. 2B). Therefore, it is evident that pilin is glycosylated and that *tfpO* is required for pilin glycosylation in *Pta* 6605.

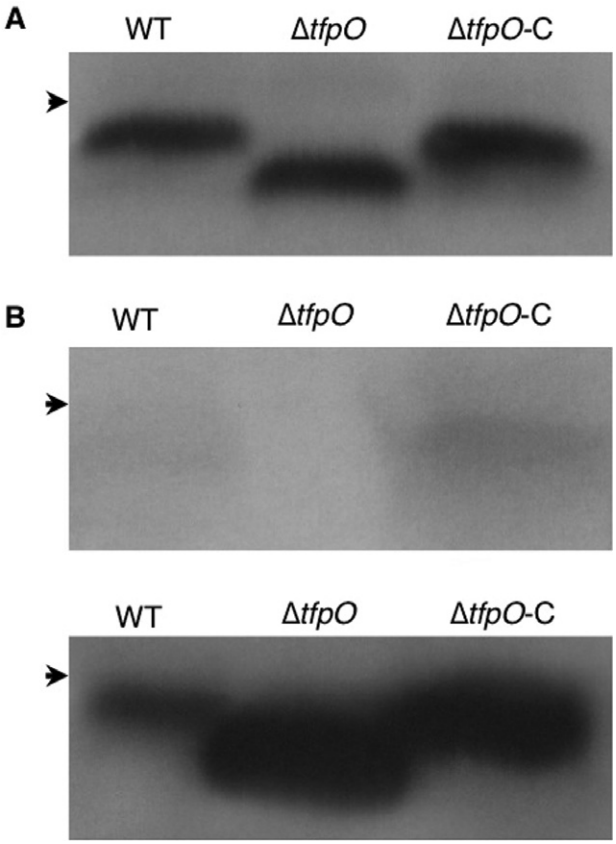

Fig. 2 Detection of the type IV pilin (PilA) protein from the wild-type (WT), its Δ*tfpO* mutant and the complemented strain of *Pseudomonas syringae* pv. *tabaci* 6605. (A) Immunoblot analysis of the PilA protein in whole-cell lysates with anti-PilA-specific antibody. (B) Staining of glycoproteins (top panel) and immunoblot analysis (bottom panel) of 1 μg of partially purified PilA protein from bacterial strains. Arrowheads indicate the positions at 15 kDa.

Involvement of flagellin glycosylation-related genes in pilin glycosylation

Flagellin and T4P are two well-known examples of bacterial glycoproteins, and we have identified three genes for flagellin glycosyltransferases (*fgt1*, *fgt2* and *vioT*) and several genes, including *vioA* and *vioB*, that are required for the biosynthesis of the precursor of the flagellin glycan, the D-Qui*p4N*(3-hydroxy-1-oxobutyl)2Me residue, in *Pta* 6605 (Ichinose *et al*., 2011; Nguyen *et al*., 2009; Taguchi *et al*., 2006; Yamamoto *et al*., 2011). To investigate the potential involvement of these genes in pilin glycosylation, we carried out SDS-PAGE and Western blot analyses using the whole-cell lysates from WT, Δ*tfpO*, Δ*fgt1*, Δ*fgt2*, Δ*vioT*, Δ*vioA* and Δ*vioB* strains in *Pta* 6605 (Fig. S1, see Supporting Information). All T4P, except that from Δ*tfpO*, showed the same mobility in SDS-PAGE analysis, indicating that *fgt1*, *fgt2*, *vioT*, *vioA* and *vioB* are not involved in pilin glycosylation.

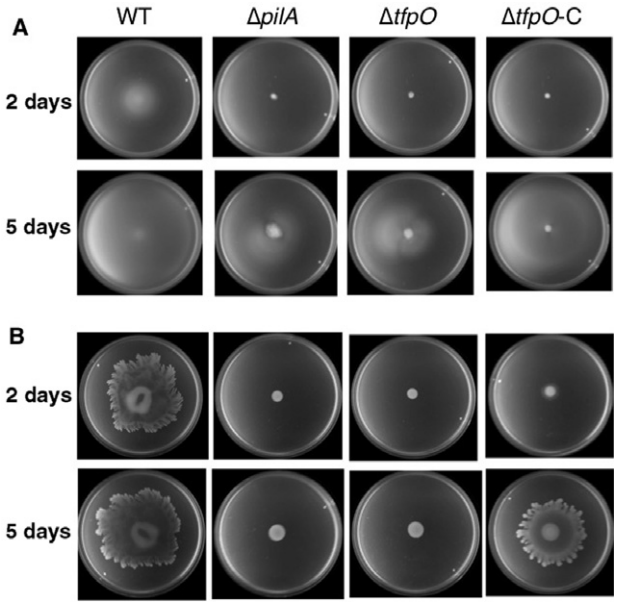

Fig. 3 Motility test. Surface swimming motility [MMMF (see Experimental procedures) with 0.25% agar] at 25 °C (A) and surface swarming motility [SWM (see Experimental procedures) with 0.45% agar] at 27 °C (B) of the wild-type (WT), Δ*pilA* and Δ*tfpO* mutants and Δ*tfpO* complemented strain. The photographs show representative results obtained from three independent experiments after incubation for 2 and 5 days.

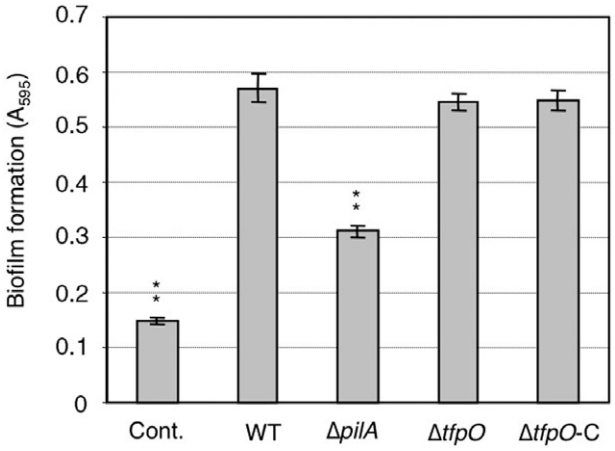

Fig. 4 Biofilm formation by the wild-type (WT), Δ*pilA* and Δ*tfpO* mutants and the Δ*tfpO* complemented strain. Each bacterium was grown in Luria–Bertani (LB) broth medium with 10 mM $MgCl_2$, transferred to fresh MMMF medium (see Experimental procedures) and incubated for 48 h at 25 °C without agitation. Averages obtained from three independent experiments are shown. Asterisks indicate a significant difference from the WT in a *t*-test (**$P < 0.005$).

Surface motility and adhesion ability of the Δ*tfpO* mutant

To determine the role of pilin glycan on bacterial behaviour, the surface swimming and swarming motilities of the mutant were examined and compared with those of the WT and nonpiliated Δ*pilA*-defective mutant strains (Fig. 3). Although no surface swimming motility was observed at 2 days after inoculation in the nonpiliated Δ*pilA* and piliated Δ*tfpO* mutant strains, that of each mutant strain was partially recovered at 5 days after inoculation. However, the surface swarming motility of Δ*pilA* and also the Δ*tfpO* mutant strain was lost even at 5 days after inoculation. Meanwhile, both surface swimming and swarming were partially restored in the Δ*tfpO*-C strain after 5 days of incubation. We have observed that the Δ*pilA* mutant retains the WT level of swimming motility in liquid culture medium (Taguchi and Ichinose, 2011). Under microscopic observation, the Δ*tfpO* mutant still exhibited swimming ability in liquid medium, similar to that of the WT strain (data not shown). These results suggest that *tfpO* is required for the surface motility of *Pta* 6605.

We also analysed the ability of each strain to form a biofilm. As observed previously, the Δ*pilA* mutant showed a reduction in biofilm formation (Taguchi and Ichinose, 2011). However, both the Δ*tfpO* and Δ*tfpO*-C strains showed similar levels of biofilm formation to the WT strain (Fig. 4), and pilin glycan did not seem to be required for biofilm formation.

Virulence of the Δ*tfpO* mutant

To investigate the effects of Δ*tfpO* mutation on the virulence towards host tobacco leaves, dip and infiltration inoculations were carried out. Although there were no significant differences in disease symptoms in plants inoculated with the WT, Δ*pilA*, Δ*tfpO* and Δ*tfpO*-C strains by the infiltration inoculation method (Fig. 5A), the disease symptoms of Δ*pilA*- and Δ*tfpO*-inoculated tobacco leaves by the dip inoculation method were reduced significantly, whereas those of the complemented strain were partially restored to the WT level (Fig. 5B). The populations of the WT strain, the Δ*pilA* and Δ*tfpO* mutant strains and the Δ*tfpO* complemented strain were examined at 1, 3 and 6 days after dip inoculation. As shown in Fig. 5C, the Δ*pilA* and Δ*tfpO* mutant strains and the Δ*tfpO*-C strain grew less than the WT strain. In the case of the Δ*tfpO*-C strain, the population was increased and the difference between the WT and Δ*tfpO*-C strain was reduced by 6 days after inoculation. This observation is consistent with the late restoration of the Δ*tfpO*-C strain in the swarming motility test. The results obtained from both experiments suggest that pilin glycan is important for surface motility and virulence.

The genetic organization of *pilA* and its surrounding region in *P. syringae*

The genomic regions around *pilA* in *P. syringae* pathovars were compared. The DNA sequence, location and direction of *pilB* (e.g. PSPPH0820 in *Pph* 1448A) and *orf* (e.g. PSPPH0825 in *Pph* 1448A) are well conserved in *P. syringae* pathovars. However, the genomic regions between *pilB* and *orf* were quite variable (Fig. 6). The *tfpO*

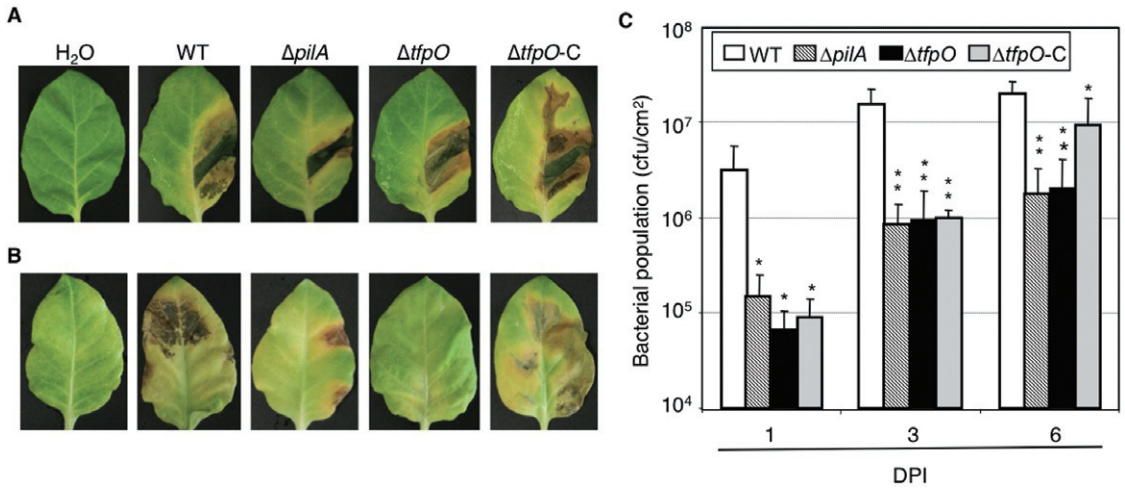

Fig. 5 Virulence assay on host tobacco plants. Tobacco leaves were inoculated with each bacterium by the infiltration method at 2×10^5 colony-forming units (cfu)/mL (A) and by the dip inoculation method at 2×10^8 cfu/mL (B), and were photographed at 9 and 12 days post-inoculation (DPI), respectively. (C) Bacterial populations were measured at 1, 3 and 6 DPI by the dip inoculation method using three tobacco leaf discs (8 mm in diameter). The bars represent standard deviations for at least three independent experiments. Asterisks indicate a significant difference from the wild-type (WT) in a *t*-test (*$P < 0.05$, **$P < 0.005$).

orthologues in *Pta* 6605 were also found in *P. syringae* pv. *mori* str. 301020, pv. *actinidiae* str. M302091, pv. *lachrymans* str. M301315, pv. *syringae* 642, pv. *maculicola* str. ES4326 and *Psav* NCPPB 3335. Their deduced amino acid sequences showed homology to $TfpO_{Pta6605}$ with 99%, 78%, 79%, 88%, 84% and 86% identities, respectively. *Pph* 1448A possesses only the C-terminal half as an incomplete fusion gene with the *ISPsy18* transposase gene. The *tfpO* gene was always located upstream from the putative polysaccharide biosynthetic gene, *psb*. However, many other strains, such as pv. *syringae* B728a, pv. *syringae* FF5, pv. *glycinea* (*Pgl*) race 4, pv. *tabaci* ATCC 11528, pv. *tomato* DC3000 and T1, and pv. *oryzae* 1_6, do not possess the *tfpO* or *psb* genes. Pathovar *syringae* B728a and pv. *aesculi* str. NCPPB3681 only possess the *pilA* gene between *pilB* and *orf1* (orthologue of PSPPH0825), and other *tfpO*-nonpossessing strains possess ORFs for glutathione *S*-transferase, transcription factor and unknown proteins. Each pathovar in *P. syringae* has been classified into five major clades by comprehensive genome sequencing analyses (Studholme, 2011). However, the distribution of the *tfpO* and *psb* genes is independent of clades and pathovars (Fig. 6).

We also analysed the DNA sequences of this *pilA*-containing region in several isolates of pv. *tabaci*, and found that isolates 7375 and MD340a possess *tfpO* and show the same genomic organization as *Pta* 6605, whereas isolates 6823 and 113R do not possess *tfpO* and show the same genomic organization as *Pta* ATCC11528 (data not shown). The deduced amino acid sequences of TfpO from pv. *tabaci* isolates 7375 and MD340a are 100% identical to that from the isolate 6605. Interestingly, the deduced amino acid sequences of PilA protein from *tfpO*-possessing strains in pv. *tabaci* are identical to each other, and those from *tfpO*-nonpossessing strains are also identical. However, PilA from *tfpO*-possessing strains in pv. *tabaci* showed only 41% identity to those from *tfpO*-nonpossessing strains. Thus, as expected, only PilA proteins from *tfpO*-possessing strains were detected with the same mobility by anti-*Pta* 6605 pilin-specific antibody (Taguchi and Ichinose, 2011), but those from *tfpO*-nonpossessing strains were not (Fig. S2, see Supporting Information). This result also suggests that the pilin proteins from strains 7375 and MD340a are glycosylated. The amino acid sequences of PilA from all *P. syringae* pathovars/strains investigated were aligned, and a phylogenetic tree was generated using the neighbour-joining (NJ) method (Saitou and Nei, 1987) (Fig. 7). We classified all PilA proteins into groups I, II, IIIa and IIIb. All PilA proteins in groups I, II and IIIa belong to *tfpO*-possessing strains, whereas all other PilA proteins in group IIIb belong to *tfpO*-nonpossessing strains, indicating that PilA proteins are at least partially divided into *tfpO*-possessing and *tfpO*-nonpossessing strains.

DISCUSSION

Function of TfpO, a pilin glycosyltransferase

In this study, we introduced a mutation into *tfpO*, a gene encoding the putative pilin glycosyltransferase in *Pta* 6605. Because the PilA protein from the WT strain, but not that from the Δ*tfpO* mutant, was positively detected by glycostaining, and because the molecular mass of the PilA protein from the Δ*tfpO* mutant was lower than that from the WT strain, TfpO was thought to be a pilin glycosyltransferase. It is known that the pilin of the *Pa* 1244 strain is glycosylated with an oligosaccharide that is structurally identical to the O-antigen repeating unit in LPS in this bacterium (Castric *et al*., 2001). It is also known that $TfpO_{Pa1244}$ catalyses the

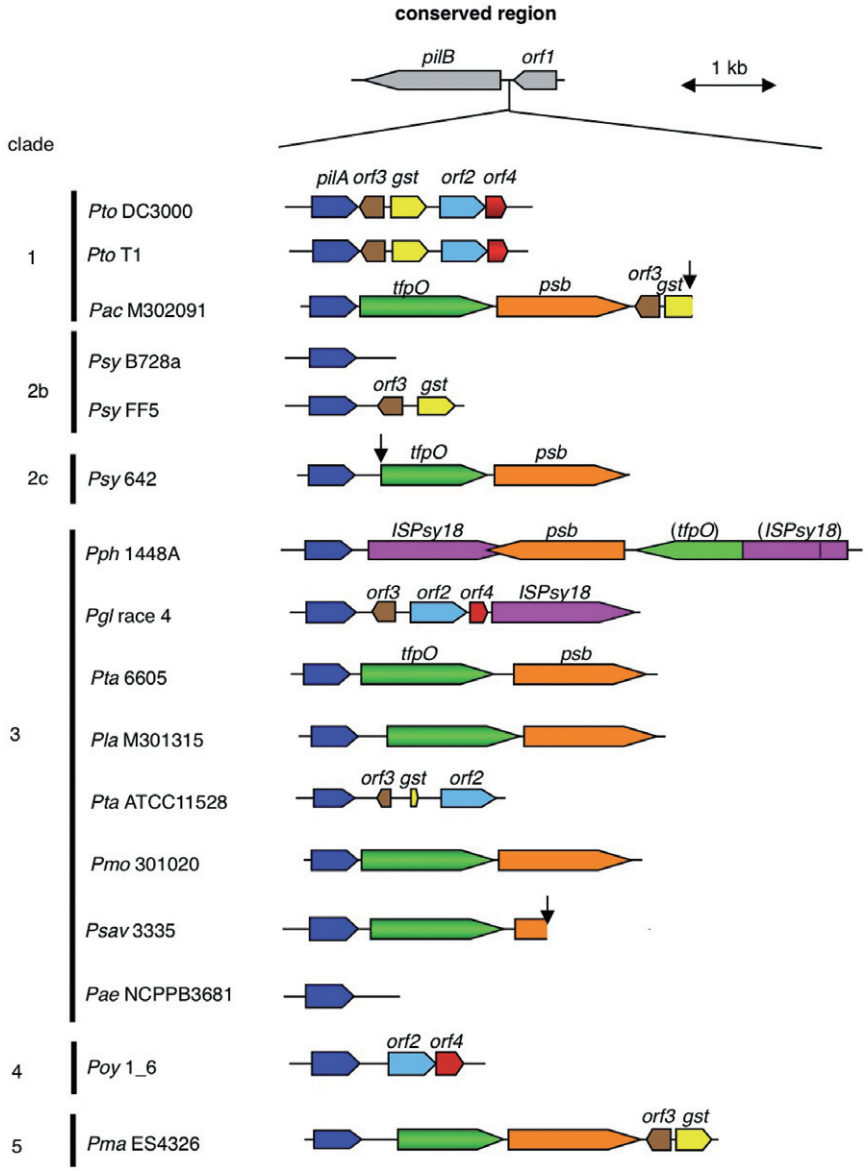

Fig. 6 Schematic organization of *pilA* and its surrounding region in *Pseudomonas syringae* pathovars. The *pilA* clusters are flanked by conserved genes encoding for type IV pilus biogenesis protein *pilB* and *orf1*. The genes having the same hypothetical function are shown in matching colours: *pilA* (blue), *tfpO* (green), *psb* (orange), *gst* (for glutathione *S*-transferase, yellow), *orf2* (light blue), *orf3* (brown), *orf4* (red) and *ISPsy18* (for ISPsy18 transposase, violet). The clusters are compared among *P. syringae* pathovars/strains whose genome sequences have been analysed so far. The abbreviations of each bacterial name and those of each Entrez Genome Project number (or RefSeq accession number where there is no Genome Project entry) are as follows: *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000, AE016853), pv. *tomato* T1 (*Pto* T1, RefSeq accession NZ_ABSM00000000), pv. *actinidiae* M302091 (*Pac* M302091, AEAL00000000), pv. *syringae* B728a (*Psy* B728a, CP000075), pv. *syringae* FF5 (*Psy* FF5, ACXZ00000000), pv. *syringae* 642 (*Psy* 642, ADGB00000000), pv. *phaseolicola* 1448A (*Pph* 1448A, CP000058), pv. *glycinea* race 4 (*Pgl* race4, AB683862), pv. *tabaci* 6605 (*Pta* 6605, AB571112), pv. *lachrymans* M301315 (*Pla* M301315, AEAF00000000), pv. *tabaci* ATCC 11528 (*Pta* ATCC11528, ACHU00000000), pv. *mori* 301020 (*Pmo* 301020, AEAG00000000), *P. savastanoi* pv. *savastanoi* NCPPB 3335 (*Psav* 3335, ADMI00000000), pv. *aesculi* NCPPB3681 (*Pae* NCPPB3681, RefSeq accession NZ_ACXS01000159), pv. *oryzae* 1_6 (*Poy* 1_6, ABZR00000000) and pv. *maculicola* ES4326 (*Pma* ES4326, AEAK00000000). The clades of *P. syringae* species were classified by the high-throughput genome sequencing approach as reported by Studholme (2011). The arrows indicate the truncated sequence contigs.

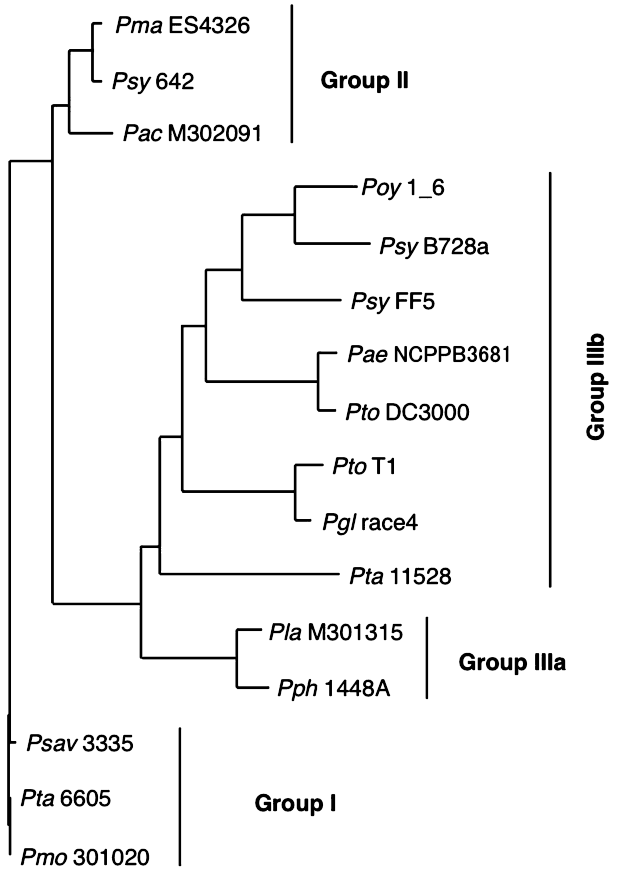

Fig. 7 Phylogenetic tree comparing the type IV pilin (PilA) protein sequences of *Pseudomonas syringae* pathovars. A phylogenetic tree was constructed from a multiple sequence alignment with the neighbour-joining method using GENETYX Ver. 15 software. The abbreviations of each bacterial strain are indicated in the legend of Fig. 6.

attachment of an O-antigen repeating unit to the β-carbon of the pilin C-terminal residue, Ser (Comer *et al*., 2002). Thus, the glycan in the pilin protein originates in the same metabolic pathway as O-antigen biosynthesis, because the mutation of the genes involved in the initial steps of O-antigen biosynthesis (either *wbpM* or *wbpL*) abolishes pilin glycosylation (DiGiandomenico *et al*., 2002). Because LPS is normally synthesized in the *Pta* 6605 Δ*tfpO* mutant strain (data not shown), TfpO is not involved in O-antigen biosynthesis in *Pta* 6605. However, the substrate specificity of $TfpO_{Pa1244}$ is extremely low (DiGiandomenico *et al*., 2002). The crucial requirement for $TfpO_{Pa1244}$ activity is the presence of a Ser residue at the C-terminus in pilin as a glycosylated site, and the pilin common sequence is not required for glycosylation (Qutyan *et al*., 2010). In contrast with $TfpO_{Pa1244}$, both TfpW from *Pa* 5196 and PglO/PglL from *Neisseria* species are able to glycosylate the internal Ser/threonine of the pilin protein (Kus *et al*., 2008; Parge *et al*., 1995; Stimson *et al*., 1995). Furthermore, the group IV PilA protein of *Pa* 5196 is more similar to the PilE pilin protein of *Neisseria* species than to those of other *P. aeruginosa* groups (Kus *et al*., 2004). $TfpO_{Pta6605}$ showed greater identity to $TfpO_{Pa1244}$ (37% over 441 residues) than $TfpW_{Pa5196}$ (19% over 225 residues) or PglL/pglO (17% over 388 residues). Therefore, the function of $TfpO_{Pta6605}$ may be more similar to that of $TfpO_{Pa1244}$ than to that of $TfpW_{Pa5196}$ or PglO/PglL.

Position of the glycosylation site and structure of pilin glycan

We revealed that the mutation of *tfpO* resulted in a reduction in the molecular mass of PilA protein by Western blot analysis in *Pta* 6605. Liquid chromatography-electrospray ionization mass analysis revealed that the molecular masses of four trypsin-digested peptide fragments in pilin protein (F1-R30, A31-K44, L45-R81 and T82-R89; the numbers indicate the positions from the N-terminus of the putative mature PilA protein) had the same values as those calculated from amino acid sequences (data not shown; F1-R30 is calculated as the peptide with a methyl group at the N-terminal phenylalanine), suggesting that these four peptides do not possess glycan. However, the remaining peptide L90-S125 was not found in the analysis. The glycosylated amino acid residue(s) should be clarified by mass spectrometric analysis of dissected PilA peptides and of the mutated PilA protein with Ser/alanine substitution.

Although the structure of the pilin glycan has not been elucidated as yet, the molecular mass of PilA was not affected in the Δ*fgt1*, Δ*fgt2*, Δ*vioT*, Δ*vioA* and Δ*vioB* mutant strains (Fig. S1). Because *fgt1*, *fgt2*, *vioT*, *vioA* and *vioB* are required for flagellin glycosylation by rhamnose and viosamine-related sugars in *Pta* 6605 (Ichinose *et al*., 2011; Nguyen *et al*., 2009; Taguchi *et al*., 2006), pilin glycan may not contain these sugars, and the pilin glycosylation system may be quite different from the flagellin glycosylation system.

Furthermore, *Pa* 1244 is known to modify its pilin with *N*-hydroxylbutyryl-*N*-formyl-Pse-xylose-*N*-acetylfucosamine, which has antigenic similarity to LPS (Castric *et al*., 2001). In a related study, the O-antigen of several *P. syringae* pv. *tabaci* distinct strains was found to be composed of three rhamnose residues and one *N*-acetylglucosamine/fucosamine residue (Zdorovenko *et al*., 1997). It should be elucidated whether or not the pilin glycan of *Pta* 6605 has the same structure as the O-antigen repeating unit.

Function of pilin glycan

Although we have clarified previously, by dip inoculation assay, that T4P of *Pta* 6605 is required for surface motility, biofilm formation and virulence on its host tobacco plant (Taguchi and Ichinose, 2011), the role of pilin glycosylation is still unclear. The glycosylation of *Neisseria* pilins at Ser63 facilitates the solubilization of pilin monomer, but does not play a role in pilus-mediated

adhesion (Marceau *et al*., 1998; Marceau and Nassif, 1999). In addition, analysis of the pilin allele distribution among isolates of *P. aeruginosa* suggests that group I pilin, which can be post-translationally glycosylated via the action of TfpO, may confer a colonization or persistence advantage in a cystic fibrosis host (Kus *et al*., 2004). The Δ*tfpO* mutant in *Pa* 1244, a representative strain of group I, has also been reported to reduce T4P-mediated twitching motility relative to the WT strain, but forms a normal biofilm (Smedley *et al*., 2005). In our study, the Δ*tfpO* mutant in *Pta* 6605 also lost all of its T4P-mediated surface swarming motility and part of its virulence, but did not lose biofilm formation ability. Although the T4P-mediated surface motility is different in *Pa* 1244 and *Pta* 6605, pilin glycan is required for these surface motilities, and its function may be similar in both bacteria.

T4P is required for twitching motility and biofilm formation in *P. aeruginosa* (Chiang and Burrows, 2003), and T4P is required for swarming motility and biofilm formation in *Pta* 6605 (Taguchi and Ichinose, 2011). Bacterial twitching motility occurs by extension, tethering and retraction of polar T4P (Mattick, 2002). Although swarming motility is different from twitching motility, the former in *Pta* 6605 also seems to require all the extension, tethering and retraction activities. Because the Δ*tfpO* mutant strain retains its biofilm formation activity, it may also retain the activities for extension and tethering and lose its retraction activity.

In *P. aeruginosa*, the T4P group I strains in which pilin is glycosylated are most prevalent in patients with cystic fibrosis (Kus *et al*., 2004), and the pilin glycan in the *Pa* 1244 strain may promote lung colonization (Smedley *et al*., 2005). Similarly, a lack of surface motility results in a reduction in virulence of the Δ*tfpO* mutant of *Pta* 6605. Overall, these data suggest that the pilus of *Pta* 6605 aids bacterial adherence to host tissue and that pilin glycan contributes to bacterial virulence through surface motility.

Co-evolution of *pilA* and *tfpO*

Previously, *P. aeruginosa* pilin was divided into five distinct phylogenetic groups (I–V) based on its amino acid sequence and the existence of unique accessory genes immediately downstream of *pilA* (Kus *et al*., 2004). Thus, it was reported that the *P. aeruginosa* pilin allele is strongly associated with cystic fibrosis isolates (Kus *et al*., 2004). However, the relationship between the intraspecies classification of *P. aeruginosa* based on the pilin allele and housekeeping genes is not known. In *P. syringae* pathovars, intrapathovar variation has been investigated by the high-throughput genome sequencing approach, and the pathovars of *P. syringae* can be classified into five major clades (Studholme, 2011). However, the genomic organization between *pilB* and *orf1* is independent of pathovars and clades, and more variable when compared with that in *P. aeruginosa*. Among 16 strains in 12 pathovars, the genomic organization around *pilA* and its surrounding region can be divided into at least nine types (three *tfpO*-possessing types and six *ftpO*-nonpossessing types), and there are many short *orfs* here (Fig. 6). Some *orfs* may encode hypothetical proteins, and some seem to be part of the disrupted gene. The existence of *tfpO*/*psb* genes is also independent of pathovars and clades, and *tfpO*/*psb* genes are distributed among pathovars/strains of *P. syringae*. For example, there are both *tfpO*-possessing and *tfpO*-nonpossessing strains in the same pathovars of *syringae* and *tabaci*. In *Pph* 1448A, the truncated *tfpO* gene and *psb* gene are placed between two transposons, both of which are *ISPsy18* genes, indicating that *tfpO* and *psb* were horizontally inserted by transposition. *Pgl* race 4 also retains *IsPsy18* in this region, indicating a horizontally active site.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Each strain of *P. syringae* pv. *tabaci* was maintained in King's B (KB) medium at 27 °C, and *Escherichia coli* strains were grown at 37 °C in Luria–Bertani (LB) medium.

Plant materials and inoculation experiments

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 26 °C with a 16-h photoperiod, and leaves of 2–3-month-old plants were used for inoculation experiments. For the dip inoculation experiments, bacterial strains cultured overnight in LB broth medium supplemented with 10 mM $MgCl_2$ were harvested after centrifugation, and then suspended in 10 mM $MgSO_4$ and 0.02% Silwet L77 (OSI Specialties, Danbury, CT, USA) at a density of 2×10^8 colony-forming units (cfu)/mL. The leaves were detached at the petiole (three leaves for each strain) and dipped in the bacterial suspension for 20 min. The leaves were then incubated in 85% humidity in a growth cabinet at 23 °C with an 18-h photoperiod and water supply. To examine the bacterial growth, five leaf discs (8 mm in diameter) were dip inoculated in the same bacterial suspension for 20 min. For the infiltration experiment, leaves (three leaves for each strain) were infiltrated with bacterial suspension in 10 mM $MgSO_4$ without Silwet at a density of 2×10^5 cfu/mL. The leaves were incubated in the same way as in the dip inoculation. We then examined the virulence in terms of symptom severity and bacterial growth. To measure the bacterial populations, dip-inoculated leaf discs were soaked in 15% H_2O_2 for 1 min to sterilize the leaf surfaces, and then washed with sterile distilled water. Then, three dip-inoculated leaf discs were ground with a mortar and pestle. The homogenates in 10 mM $MgSO_4$ were plated on KB plates after serial dilutions. After 48 h of incubation at 27 °C, the colonies were counted and the bacterial populations were calculated.

DNA cloning and the generation of mutant and complemented strains

To investigate the genomic information on the *pilA* region in *Pta* strains, two primers, pilA-F (5′-GTTGACTTCTTCAAGGCTGG-3′) and pilA-R (5′-

Table 2 Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli strain		
DH5a	*F–λ–ø80dLacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK–mK+) supE44 thi-1gyrA relA1*	Takara, Kyoto, Japan
S17-1	*thi pro hsdR–hsdM + recA [chr::RP4-2-Tc::Mu-Km::Tn7]*	Schäfer *et al*. (1994)
S17-1 λ-pir	*λ pir lysogen of S17 (Tpr Smr thi pro hsdR-M + recA RP::2-Tc::Mu-Km::Tn7*	Simon *et al*. (1983)
Pseudomonas syringae pv. *tabaci*		
Isolate 6605	Wild-type, Nal^r	Taguchi *et al*. (2001)
6605-Δ*pilA*	Isolate 6605 Δ*pilA*, Nal^r	Taguchi and Ichinose (2011)
6605-Δ*tfpO*	Isolate 6605 Δ*tfpO*, Nal^r	This study
6605-Δ*tfpO*-C	Isolate 6605 Δ*tfpO* complemented, Nal^r (pBSL*tfpO*)	This study
Isolate 6823	Wild-type	Taguchi *et al*. (2001)
Isolate 7375	Wild-type	Taguchi *et al*. (2001)
Isolate MD340a	Wild-type	Gift from Dr J. Lydon
Isolate 113R	Wild-type	Gift from Dr J. Lydon
Isolate ATCC 11528	Wild-type	Rico and Preston (2008)
Plasmid		
pCR-Blunt II-TOPO	Cloning vector, Km^r	Invitrogen
pTOPO-*pilA*	5533-bp insert containing *pilA* surrounding, Km^r	This study
pGEM-T easy	Cloning vector, Amp^r	Promega, Madison, WI, USA
pGEM-*tfpO*	3704-bp insert containing *pilA* and *tfpO*, Amp^r	This study
pGEM-Δ*tfpO*	3204-bp insert containing *pilA* and 500-bp interval deleted *tfpO*, Amp^r	This study
pGEM-*tfpO*-C	2339-bp insert containing *pilA* and *tfpO*, Amp^r	This study
pK18*mobsacB*	Small mobilizable vector, Km^r, sucrose-sensitive (*sacB*)	Schäfer *et al*. (1994)
pK18-Δ*tfpO*	3204-bp insert through *Eco*RI site containing *pilA* and 500-bp interval deleted *tfpO*, Km^r	This study
pBSL118	Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp^r, Km^r	Alexeyev *et al*. (1995)
pBSL*tfpO*-C	2339-bp insert containing *pilA* and *tfpO*, Amp^r, Km^r	This study

Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance.

CCCGTGGTACTTCAAATTCG-3′), were designed on the basis of two conserved genes, *pilB* (PSPPH_0820) and *orf1* (PSPPH_0825), in the registered sequences of *Pph* 1448A (accession number CP000058). The resulting PCR products were cloned into pCR-Blunt II-TOPO plasmid, and DNA sequencing was performed using an ABI PRISM 3130xl (Applied Biosystems, Chiba, Japan) and a BigDye Terminator Cycle Sequencing Kit.

To generate the Δ*tfpO* mutant strain, a pair of primers (5′-GT CAGCGTCACTCTGAGTAG-3′ for tfpO-F and 5′-AACGCACCAATGGCA AAAGC-3′ for tfpO-R) was used to amplify the DNA region containing *tfpO* with PCR, and the DNA product was subsequently isolated by the pGEM-T-Easy vector cloning system. Digestion was performed with *Bsm*I to delete an internal 500-bp region for the generation of the mutant plasmid. The internally deleted DNA fragment was excised with *Eco*RI digestion and introduced into an *Eco*RI site of the pK18*mobsacB* plasmid (Schäfer *et al*., 1994). Deletion mutant strains were obtained by conjugation and homologous recombination according to the methods reported previously (Taguchi *et al*., 2006).

For complementation of the Δ*tfpO* mutant, the primers 5′-CA GACATATAACCACCAGAC-3′ for C-tfpO-F and 5′-TGTAACCAGCGTAA TTGCGC-3′ for C-tfpO-R were used to amplify the predicted promoter region and the entire *pilA* and *tfpO* ORFs. The amplified DNA fragment was inserted into the *Eco*RI sites of pBSL118, a transposon vector (Alexeyev *et al*., 1995), to generate pBSL*tfpO*-C, and introduced into the Δ*tfpO* mutant by conjugation using the *E. coli* S17-l λpir strain to generate Δ*tfpO*-C. All sequences of recombinant DNA were confirmed by an ABI PRISM 3130xl using a BigDye Terminator Cycle Sequencing Kit.

The nucleotide sequences of the DNA fragments for the region from *pilB* to *orf1* for *Pta* 6605, MD340a, 7375, 113R, 6823 and *Pgl* race 4 have been deposited under the respective accession numbers AB571112, AB677536, AB677537, AB677538, AB677539 and AB683862 in the DDBJ, EMBL and GenBank nucleotide sequence databases.

Motility assay

Bacteria cultured overnight in LB medium containing 10 mM $MgCl_2$ at 25 °C were resuspended in 10 mM $MgSO_4$ and adjusted to an optical density at 600 nm (OD_{600}) of 0.1. For the assessment of surface motilities on semi-solid agar medium, 3-μL aliquots were inoculated in the centre of 0.25% agar MMMF plates [50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$ and 1.7 mM NaCl, pH 5.7, supplemented with 10 mM each of mannitol and fructose] for the swimming assay and of 0.45% agar SWM plates (0.5% peptone, 0.3% yeast extract) for the swarming assay, as described in Taguchi *et al*. (2006). The motility was examined using three plates for each strain, and was observed 2 and 5 days after incubation at 25 °C for the swimming assay and at 27 °C for the swarming assay. The swimming motility in liquid MMMF medium was observed using a phase-contrast microscope.

Biofilm formation

Each bacterial strain was grown in LB broth medium containing 10 mM $MgCl_2$ overnight, and the concentration was adjusted to $OD_{600} = 0.1$ with 3 mL of fresh MMMF medium in a polystyrene tube (three tubes for each strain). After 48 h of incubation at 25 °C without agitation, adherent bacteria were stained with 0.5% crystal violet for 1 h, and loosely bound bacteria were removed by washing with distilled H_2O three times. For quantitative analysis of the biofilms, crystal violet was extracted from

stained cells by 3 mL of 95% ethanol, and OD_{595} values were measured (Taguchi *et al*., 2006).

Preparation of the pilus-containing surface fraction

Pili were partially purified as described previously (Taguchi and Ichinose, 2011) with a minor modification. Each bacterium was grown on 1.5% agar KB plates for 48 h at 27 °C. Cells from 20 plates were gently scraped off the agar surface and resuspended in 200 mL of phosphate buffer (50 mM sodium phosphate, pH 6.8). Pili were sheared off from the cells by stirring for 30 min, and cells were removed by centrifugation at 10 000 ***g*** for 20 min. After filtration of the supernatant through a 0.45-μm pore filter, 30% polyethylene glycol (PEG8000) and 5 M NaCl were added to final concentrations of 3% and 0.5 M, respectively. The mixture was incubated at 4 °C overnight to allow the precipitation of proteins. Pilus-containing fractions were obtained as pellets by centrifugation at 100 000 ***g*** for 30 min, and were resolved in water. The dissolved pilin fraction was loaded through Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific, Yokohama, Japan) to avoid the contamination of LPS and exopolysaccharide, and was then re-extracted after the appropriate band of pilin had been cut from 15% SDS-PAGE.

Western blot analysis and detection of glycoproteins

Bacterial whole-cell lysate was prepared from bacterial cells harvested from an overnight culture in KB broth medium with shaking at 27 °C. PilA proteins in bacterial whole-cell lysates or pilus-containing surface fractions were separated on a 15% SDS-PAGE gel. After transfer to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), the proteins were subjected to Western blot analysis using a rabbit polyclonal anti-$PilA_{Pta6605}$-specific peptide antibody with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA) and an alkaline phosphatase-based chemiluminescent detection system (CDP-Star Reagent, New England Biolabs, Ipswich, MA, USA), as described previously (Taguchi and Ichinose, 2011). The anti-$PilA_{Pta6605}$-specific antibody was generated with a synthetic peptide (VAENYSNGSAQADVC).

The partially purified pilin proteins from *Pta* 6605 were separated by SDS-PAGE, and then subjected to staining with a GelCode® Glycoprotein Staining Kit (Pierce, Rockford, IL, USA), which specifically stains glycoproteins as magenta bands on SDS-PAGE gel.

Statistical analysis

The results of the biofilm formation assay and the bacterial populations in host tobacco leaves are expressed as means with standard deviations (SDs). The two-tailed *t*-test was performed for comparisons between the quantitative measurement of the WT, each mutant and the complemented strains. Values of $P < 0.05$ were considered to be statistically significant.

ACKNOWLEDGEMENTS

We would like to thank the Leaf Tobacco Research Laboratory of Japan Tobacco Inc.; Dr John Lydon (Sustainable Agricultural Systems Laboratory, US Department of Agriculture); and Dr Gail Preston (Department of Plant Sciences, University of Oxford, UK) for providing *Pta* strains 6605, 6823 and 7375; 113R and MD340a; and ATCC11528, respectively. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

REFERENCES

Aas, F.E., Vik, A., Vedde, J., Koomey, M. and Egge-Jacobsen, W. (2007) *Neisseria gonorrhoeae* O-linked pilin glycosylation: functional analyses define both the biosynthetic pathway and glycan structure. *Mol. Microbiol*. **65**, 607–624.

Alexeyev, M.F., Shokolenko, I.N. and Croughan, T.P. (1995) New mini-Tn5 derivatives for insertion mutagenesis and genetic engineering in gram-negative bacteria. *Can. J. Microbiol*. **41**, 1053–1055.

Bahar, O., Goffer, T. and Burdman, S. (2009) Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. *Mol. Plant–Microbe Interact*. **22**, 909–920.

Benz, I. and Schmidt, M.A. (2002) Never say never again: protein glycosylation in pathogenic bacteria. *Mol. Microbiol*. **45**, 267–276.

Burdman, S., Bahar, O., Parker, J.K. and Fuente, L. (2011) Involvement of type IV pili in pathogenicity of plant pathogenic bacteria. *Genes* **2**, 706–735.

Castric, P. (1995) *pilO*, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology*, **141**, 1247–1254.

Castric, P., Cassels, F.J. and Carlson, R.W. (2001) Structural characterization of the *Pseudomonas aeruginosa* 1244 pilin glycan. *J. Biol. Chem*. **276**, 26 479–26 485; correction: **38**, 36058.

Chiang, P. and Burrows, L.L. (2003) Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J. Bacteriol*. **185**, 2374–2378.

Comer, J.E., Marshall, M.A., Blanch, V.J., Deal, C.D. and Castric, P. (2002) Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect. Immun*. **70**, 2837–2845.

DiGiandomenico, A., Matewish, M.J., Bisaillon, A., Stehle, J.R., Lam, J.S. and Castric, P. (2002) Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: specificity of glycan substrate. *Mol. Microbiol*. **46**, 519–530.

Harvey, H., Kus, J.V., Tessier, L., Kelly, J. and Burrows, L.L. (2011) *Pseudomonas aeruginosa* D-arabinofuranose biosynthetic pathway and its role in type IV pilus assembly. *J. Biol. Chem*. **286**, 28 128–28 137.

Ichinose, Y., Shimizu, R., Ikeda, Y., Taguchi, F., Marutani, M., Mukaihara, T., Inagaki, Y., Toyoda, K. and Shiraishi, T. (2003) Need for flagella for complete virulence of *Pseudomonas syringae* pv. *tabaci*: genetic analysis with flagella-defective mutants Δ*fliC* and Δ*fliD* in host tobacco plants. *J. Gen. Plant Pathol*. **69**, 244–249.

Ichinose, Y., Taguchi, F., Nguyen, L.C., Naito, K., Suzuki, T., Inagaki, Y., Toyoda, K. and Shiraishi, T. (2011) Glycosylation of bacterial flagellins and its role in motility and virulence. In: *Genome-Enabled Analysis of Plant–Pathogen Interactions* (Wolpert, T., Shiraishi, T., Collmer, A., Glazebrook, J. and Akimitsu, K., eds), pp. 215–224. St. Paul, MN: APS Press.

Kus, J.V., Tullis, E., Cvitkovitch, D.G. and Burrows, L.L. (2004) Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology*, **150**, 1315–1326.

Kus, J.V., Kelly, J., Tessier, L., Harvey, H., Cvitkovitch, D.G. and Burrows, L.L. (2008) Modification of *Pseudomonas aeruginosa* Pa5196 type IV pilins at multiple sites with D-Araf by a novel GT-C family arabinosyltransferase, TfpW. *J. Bacteriol*. **190**, 7464–7478.

Logan, S.M. (2006) Flagellar glycosylation—a new component of the motility repertoire? *Microbiology*, **152**, 1249–1262.

Marceau, M. and Nassif, X. (1999) Role of glycosylation at Ser63 in production of soluble pilin in pathogenic *Neisseria*. *J. Bacteriol*. **181**, 656–661.

Marceau, M., Forest, K., Beretti, J.L., Tainer, J. and Nassif, X. (1998) Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion. *Mol. Microbiol*. **27**, 705–715.

Mattick, J.S. (2002) Type IV pili and twitching motility. *Annu. Rev. Microbiol*. **56**, 289–314.

Nguyen, L.C., Yamamoto, M., Ohnishi-Kameyama, M., Andi, S., Taguchi, F., Iwaki, M., Yoshida, M., Ishii, T., Konishi, T., Tsunemi, K. and Ichinose, Y. (2009) Genetic analysis of the genes involved in the synthesis of modified 4-amino-4,6-dideoxyglucose in flagellin of *Pseudomonas syringae* pv. *tabaci*. *Mol. Genet. Genomics*, **282**, 595–605.

Nudleman, E. and Kaiser, D. (2004) Pulling together with type IV pili. *J. Mol. Microbiol. Biotechnol*. **7**, 52–62.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.E., Getzoff, E.D. and Tainer, J.A. (1995) Structure of the fibre-forming protein pilin at 2.6 A resolution. *Nature*, **378**, 32–38.

Power, P.M. and Jennings, M.P. (2003) The genetics of glycosylation in Gram-negative bacteria. *FEMS Microbiol. Lett.* **218**, 211–222.

Power, P.M., Seib, K.L. and Jennings, M.P. (2006) Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to *wzy*-dependent O-antigen biosynthesis in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **347**, 904–908.

Qutyan, M., Henkel, M., Horzempa, J., Quinn, M. and Castric, P. (2010) Glycosylation of pilin and nonpilin protein constructs by *Pseudomonas aeruginosa* 1244. *J. Bacteriol.* **192**, 5972–5981.

Rico, A. and Preston, G.M. (2008) *Pseudomonas syringae* pv. *tomato* DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Mol. Plant–Microbe Interact.* **21**, 269–282.

Roine, E., Raineri, D.M., Romantschuk, M., Wilson, M. and Nunn, D.N. (1998) Characterization of type IV pilus genes in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant–Microbe Interact.* **11**, 1048–1056.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Schäfer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. and Puhler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, **145**, 69–73.

Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology*, **1**, 784–791.

Smedley, J.G. III, Jewell, E., Roguskie, J., Horzempa, J., Seyboldt, A., Stolz, D.B. and Castric, P. (2005) Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *J. Bacteriol.* **73**, 7922–7931.

Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H.R., Payne, G., Saunders, J.R., Jennings, M.P., Barker, S. and Panico, M. (1995) Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol. Microbiol.* **17**, 1201–1214.

Strom, M.S. and Lory, S. (1993) Structure–function and biogenesis of the type IV pili. *Annu. Rev. Microbiol.* **47**, 565–596.

Strom, M.S., Nunn, D.N. and Lory, S. (1993) A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. *Proc. Natl. Acad. Sci. USA*, **90**, 2404–2408.

Studholme, D.J. (2011) Application of high-throughput genome sequencing to intrapathovar variation in *Pseudomonas syringae*. *Mol. Plant Pathol.* **12**, 829–838.

Taguchi, F. and Ichinose, Y. (2011) Role of type IV pili in virulence of *Pseudomonas syringae* pv. *tabaci* 6605: correlation of motility, multidrug resistance, and HR-inducing activity on a nonhost plant. *Mol. Plant–Microbe Interact.* **24**, 1001–1011.

Taguchi, F., Tanaka, R., Kinoshita, S., Ichinose, Y., Imura, Y., Andi, S., Toyoda, K., Shiraishi, T. and Yamada, T. (2001) $Harpin_{Psta}$ from *Pseudomonas syringae* pv. *tabaci* is defective and deficient in its expression and HR-inducing activity. *J. Gen. Plant Pathol.* **67**, 116–123.

Taguchi, F., Takeuchi, K., Katoh, E., Murata, K., Suzuki, T., Marutani, M., Kawasaki, T., Eguchi, M., Katoh, S., Kaku, H., Yasuda, C., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2006) Identification of glycosylation genes and glycosylated amino acids of flagellin in *Pseudomonas syringae* pv. *tabaci*. *Cell. Microbiol.* **8**, 923–938.

Taguchi, F., Shibata, S., Suzuki, T., Ogawa, Y., Aizawa, S., Takeuchi, K. and Ichinose, Y. (2008) Effects of glycosylation on swimming ability and flagella polymorphic transformation of *Pseudomonas syringae* pv. *tabaci* 6605. *J. Bacteriol.* **190**, 764–768.

Taguchi, F., Takeuchi, K., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2009) Glycosylation of flagellin from *Pseudomonas syringae* pv. *tabaci* 6605 contributes to evasion of host tobacco plant surveillance system. *Physiol. Mol. Plant Pathol.* **74**, 11–17.

Virji, M., Saunders, J.R., Sims, G., Makepeace, K., Maskell, D. and Ferguson, D.J.P. (1993) Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Mol. Microbiol.* **10**, 1013–1028.

Voisin, S., Kus, J.V., Houliston, S., St-Michael, F., Watson, D., Cvitkovitch, D.G., Kelly, J., Brisson, J.R. and Burrows, L.L. (2007) Glycosylation of *Pseudomonas aeruginosa* strain Pa5196 type IV pilins with mycobacterium-like α-1,5-linked D-Ara*f* oligosaccharides. *J. Bacteriol.* **189**, 151–159.

Yamamoto, M., Ohnishi-Kameyama, M., Nguyen, L.C., Taguchi, F., Chiku, K., Ishii, T., Ono, H., Yoshida, M. and Ichinose, Y. (2011) Identification of genes involved in the glycosylation of modified viosamine of flagellins in *Pseudomonas syringae* by mass spectrometry. *Genes*, **2**, 788–803.

Zdorovenko, G.M., Solyanik, L.P., Yahovleva, L.M. and Paramonov, N.A. (1997) Characterization of O-antigens from different strains of *Pseudomonas syringae* pv. *tabaci*. *Biochemistry (Moscow)*, **62**, 28–37.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Detection of type IV pilin (PilA) protein. Anti-$pilA_{Pta6605}$-specific antibody was used to detect PilA protein in the whole-cell lysates from wild-type (WT), Δ*fgt1*, Δ*fgt2*, Δ*vioT*, Δ*vioA* and Δ*vioB* strains in *Pseudomonas syringae* pv. *tabaci*. Arrowheads indicate the positions at about 15 kDa.

Fig. S2 Detection of type IV pilin (PilA) protein. Anti-$pilA_{Pta6605}$-specific antibody was used to detect PilA protein in the whole-cell lysates from several isolates of *Pseudomonas syringae* pv. *tabaci*. Arrowheads indicate the positions at about 15 kDa.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.