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Effects of glycosylation on swimming ability and flagellar polymorphic transformation in Pseudomonas syringae pv. tabaci 6605

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Abstract The role of flagellin glycosylation on motility was investigated in *Pseudomonas syringae* pv. *tabaci*. The swimming activity of glycosylation-defective mutants was prominently decreased in a highly viscous medium. The mutants showed differences in polymorphic transitions and in bundle formation of flagella, indicating that glycosylation stabilizes the filament structure and lubricates rotation of the bundle.

Pseudomonas syringae pv. *tabaci* 6605 is a phytopathogenic bacterium that causes wildfire disease in tobacco plants (10, 26). The cell possesses several flagella at the cell pole when grown in liquid cultures. Our previous study revealed that the flagella of this bacterial strain are indispensable for intrinsic virulence on the host tobacco plant and that flagellin, the major component protein of the flagellum, is a major elicitor of hypersensitive cell death in non-host plants (10, 23, 26-28). Furthermore, the flagellin of *P. syringae* was found to be glycosylated at six serine residues by the products of the *orf1* and *orf2* genes that are located in a glycosylation island (26, 29, 30). Recently, the *orf1* and *orf2* genes in the *P. syringae* glycosylation island have been renamed *fgt1* (flagellar glycosyltransferase 1) and *fgt2*, respectively. To examine the roles of glycosylation in bacterial virulence and interactions with plants, we constructed a glycosylation-defective mutant (Δ*fgt1*), a partially defective mutant (Δ*fgt2*), single Ser/Ala-substituted mutants (S143A, S164A, S176A, S183A, S193A, and S201A) and a six serine-substituted mutant (6 S/A) (26). Using these mutants, we demonstrated that glycosylation of flagellin is required for virulence towards host tobacco plants and swarming and adhesion abilities; thus, glycosylation may play an important role in determining host specificity (26).

In this study, swimming ability, polymorphic flagellar transitions at various pH and salt concentrations, and bundle formation were analyzed to compare the structural and functional differences between flagella of the wild type (WT) and glycosylation-defective

mutants.

Effect of viscosity on swimming of WT and glycosylation-defective mutants.

WT and mutant strains were grown in LB media supplemented with 10 mM $MgCl_2$ with vigorous agitation at 25°C for 24 h. The overnight cultures were left standing without agitation for another 6 h. While WT cells remained in suspension, the Δ*fgt1* and 6 S/A mutant cells were precipitated (Fig. 1). To investigate the cause of this phenomenon, the proportions of swimming cells in the supernatant and precipitate from each sample were compared. The swimming bacteria were observed by phase contrast microscopy equipped with a video recording system. Approximately 200 cells were counted to calculate the percentage of swimming cells. More than 60% of WT cells in suspension culture swam, whereas 18% of Δ*fgt1* and 19% of 6 S/A mutant cells in the supernatant of each culture did. Furthermore, only 6% of Δ*fgt1* and 7% of 6 S/A mutant cells in culture precipitates swam. These results might indicate that a defect of swimming ability in these mutants causes the aggregation of cells.

For a more precise investigation of the ability of the flagella to propel the cell in liquid culture, the effect of viscosity was examined by conventional phase contrast microscopy according to the previously reported method (3). Cells were cultured overnight in LB medium supplemented with 10 mM $MgCL_2$ and inoculated into MMMF minimal medium (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 mannitol and fructose and cultured at 23°C for 24 h. The viscosity was increased by addition of polyvinylpyrrolidone K 90 (PVP; Wako Pure Chemical Industries) to the MMMF culture medium. As shown in Fig. 2A, approximately 83% of WT cells swam in the absence of PVP, while about 65-75% of cells from the Δ*fgt1*, Δ*fgt2*, six serine-substituted (6 S/A), S176A, and S183A mutants swam. In the presence of 2% PVP, the percentages of swimming cells of all bacterial strains except the WT were decreased. In the presence of 6% PVP, the rates of swimming cells of the WT and four single

serine-substituted mutants (S143A, S164A, S193A, and S201A) were 50-55%, and those of other mutant strains (Δ*fgt1*, Δ*fgt2*, 6 S/A, S176A, and S183A) were decreased to about 30-38%. We previously reported that Δ*fgt1* (Δ*orf1*), Δ*fgt2* (Δ*orf2*), 6 S/A, S176A, and S183A mutants were impaired in pathogenicity on host tobacco plants and reduced adhesion and swarming abilities on SWM plate (0.5% agar, 0.5% peptone, 0.3% yeast extract) (26). The lower swimming ability of the glycosylation-defective mutants in viscous medium may be one of the causes of these phenotypes.

The swimming speed was calculated by tracing the tracks of individual bacteria recorded on videotape and measuring the distance traveled in a period of time (3). The swimming speed profiles against viscosity of WT and mutant strains are shown in Fig. 2B. In the absence of PVP, WT cells swam in MMMF medium at 83 μm/s, four single serine-substituted mutants (S143A, S164A, S193A, and S201A) swam at 70-76 μm/s, and the other mutants (Δ*fgt1*, Δ*fgt2*, 6 S/A, S176A, and S183A) swam at 59-69 μm/s. In 2% PVP, the swimming speed of all bacterial strains was slightly decreased. The viscosity effect was more prominent in 6% PVP; the cell swimming speeds of WT, four single serine-substituted mutants (S143A, S164A, S193A, and S201A) and the Δ*fgt1*, Δ*fgt2*, 6 S/A, S176A, and S183A mutants were 45 μm/s, 24-28 μm/s, and 17-23 μm/s, respectively.

Because the reductions of the percentage of swimming cells and the swimming speed might be due to a regulatory effect on gene expression, we performed an immunoblot analysis to measure flagellin protein accumulation. Each overnight culture (LB with 10 mM $MgCl_2$) was centrifuged, and the concentration of cells was adjusted to 2 x 10^8 cfu ml^{-1}. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis, and an antibody that was raised against purified flagellin from *P. syringae* pv. *tabaci* was used (27). However, the amounts of flagellin protein from each mutant were almost identical (data not shown), indicating that there are no significant difference in flagella numbers in WT and

glycosylation-defective mutant strains.

Polymorphic transition and bundle formation of flagellar filaments from WT and glycosylation-defective mutants.

The bacterial flagellum is a filament consisting of flagellin protein, and the helical shape, which is defined by the helical parameters of pitch (P) and diameter (D), is essential for movement. Despite the different primary structures of flagellins, flagellar helices are similar among the same family (22). In peritrichously flagellated species such as *Escherichia coli* and *Salmonella typhimurium,* the left-handed helical filament named "Normal" is the common form in smoothly swimming cells, and the right-handed form called "Curly" appears only transiently during cell tumbling (16). These two shapes are reversibly converted under various environmental conditions such as changes in pH, salt concentration, and temperature. Other polymorphs include "Coiled" and "Semi-Coiled", which are not very effective for movement. In an extreme case, the Straight form was found in non-motile mutants with amino acid substitutions (18). However, in polar flagellated species such as the marine bacterium *Idiomarina loihiensis* and *Pseudomonas aureginosa*, the helical parameters are smaller than those of peritrichously flagellated species. For example, the Normal form of the polar flagellum is similar to the Curly form of the peritrichous flagellum in pitch and diameter but is left-handed. (22). We categorized the left-handed curly-like filaments as small-Normal (S-Normal), and assumed that *P. syringae* pv. *tabaci* flagella might belong to this flagellar group.

To compare the nature of flagellar filaments of WT and glycosylation-defective mutants of *P. syringae* pv. *tabaci* 6605, the helical parameters of each polymorph were measured, as shown in Table 1. The polymorphic transitions due to changes in pH and salt concentration were examined as described by Kamiya and Asakura (13). Flagellar filaments were purified as described previously (22). The polymorphic shapes of filaments observed by

dark field light microscopy and diagrams of the polymorphs observed are shown in Fig. 3A and 3B. At low pH (pH 5.0-7.0), the shapes of flagella filaments were dominantly Semi-Coiled, and S-Normal (left-handed Curly-like) filaments were also found at a low NaCl concentration (0.1 M). When the pH was further shifted to acidic, the filaments were changed to the Coiled form and then the Straight form. At pH 3.0 and 0.1 M NaCl concentration, flagella filaments were depolymerized.

On the other hand, various abnormal shapes of flagella filaments were observed in the Δ*fgt1* and 6 S/A mutants between pH 4.0 to 7.0 in a wide range of salt concentrations. These results suggest that the filaments from non-glycosylated mutants show no distinct polymorphic forms and do not take on proper polymorphs in response to the change of environmental conditions. Because single filaments of the non-glycosylated mutant showed different shapes at the same time, we called them “undulant” filaments. When the pH was further shifted to acidic, filaments from both mutants changed to the Straight form and then were depolymerized. In the cases of single Ser/Ala-substituted mutants, the polymorphic transition of flagella filaments was similar to that of the WT. Moreover, there was no correlation between the polymorphic transition and viscosity in flagella filaments from both WT and mutants (data not shown).

We suspected that undulant filaments from non-glycosylated mutants might be structurally unstable and, thus, measured the amounts of unpolymerized flagellin present in the spent medium. The protein from the supernatant of overnight cultures of each strain was precipitated by the addition of trichloroacetic acid at a final concentration of 10% (w/v) and dissolved in 1/100 of the original volume of PBST buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, and Tween-20, pH 7.4). The immunoblot analysis revealed no significant difference in the amounts of intact flagellin from each mutant and WT strain. Furthermore, we did not detect broken filaments in the spent media by electron microscopy

(data not shown). These results suggest that the filament formation of glycosylation-defective mutants was normal.

Peritrichous flagella form a bundle when the cell is swimming smoothly. The bundle formation of flagella on WT and non-glycosylated mutant cells was compared by dark field microscopy. The shapes of flagella have been reported as bright particles with twisted bundles when seen by dark field microscopy under strong illumination (19). Using this method, bundled flagella were observed only on slowly moving or resting cells. The flagellar bundles on WT cells were too loose to observe, while irregular flagellar bundles were constantly observed in Δ*fgt1* and 6 S/A mutants (Fig. 4A). The binding between filaments appeared tight on electron microscopy, although this seldom occurs in WT cells (Fig.4B). We also examined the bundle formation using flagella ejected from WT and non-glycosylated mutants, followed by PEG precipitation (22). Many thick flagellar bundles were observed only in preparations of the mutants. Furthermore, bundled flagella were also found frequently in the precipitated samples from non-glycosylated mutants shown in Fig. 1 by dark field microscopy and electron microscopy (data not shown). These results suggested that the surface charge or hydrophobic properties of the filament of the non-glycosylation mutants might be changed so that more than two filaments interact tightly along their length. This irregular entanglement of filaments would result in a reduction of swimming ability in these mutants.

Glycosylation of the surface structure has been reported for pili (6, 7), for S-layers (24), and for flagella (1, 5, 8, 9, 12, 27, 29, 31). In gram-negative bacteria, glycosylation has been shown to play important roles in adhesion (4, 14), solubility (17), immune response (2, 20, 25, 32), motility, and flagella filament assembly (21). Further, it was also pointed out that glycosylation of flagellin in Archae may increase the structural stability of the filament and its resistance to proteolysis (15). We previously demonstrated that all the glycosylation sites

of the flagellin molecule in *P. syringae* pv. *tabaci* 6605 are located on the putative surface-exposed region and that glycosylation might be involved in pathovar-specific recognition (11, 26, 30). This exposed surface region is also considered to be a major antigen for the adaptive immune system in mammals (33). Very recently, we identified the glycan structure at serine 201 (S201) of flagellin from *P. syringae* pv. *tabaci* and pv. *glycinea* by sugar composition analysis, mass spectrometry, and ^{1}H and ^{13}C NMR spectroscopy. The S201 glycan was composed of an unique trisaccharide consisting of two rhamnosyl residues and one modified 4-amino-4,6-dideoxyglucosyl residue (29). Further analysis to elucidate the glycan structure is in progress.

The results obtained in this study revealed that flagellin glycosylation facilitates proper flagellar suprastructures that contribute to the proper swimming ability of the bacterium. The regular transitions of flagella morphology indicate that glycosylation of the filament surface increases the slippage between filaments and contributes to smooth swimming. Previously we found that glycosylation of flagellin is required for bacterial virulence (26, 30). The reduction of motility eventually impairs the virulence of glycosylation-defective mutants. In nature, flagellin glycosylation may enhance the swimming ability on the viscous and sticky surface of tobacco leaves. Together with our previous results, it is likely that glycosylation of flagellin in *P. syringae* pv. *tabaci* 6605 is indispensable for virulence on the host tobacco plant.

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Table 1. Helical parameters of flagella filaments of *P. syringae*

Polymorphic form	Handedness	Pitch (μm)	Diameter (μm)
Normal	Left	1.59	0.18
Semi-Coiled	Left	1.49	0.39
Coiled	Left	1.04	0.65

Figure legends

FIG. 1. Two-night culture of wild-type (WT), *fgt1*-deleted mutant (Δ*fgt1*), and six serine-substituted mutants (6 S/A). Bacterial strains were incubated in LB supplemented with 10 mM $MgCl_2$ for 24 h at 25°C with agitation, then for 20 h without agitation.

FIG. 2. (A) Effect of viscosity on swimming motility of WT and glycosylation-defective mutants (Δ*fgt1*, *fgt2*-deleted mutant (Δ*fgt2*), 6S/A, and 6 strains of single Ser/Ala-substituted mutants (S143A, S164A, S173A, S183A, S193A, and S201A) from *P. syringae* pv. *tabaci* 6605. The percentage of swimming cells is indicated. Viscosity was increased by the addition of polyvinylpyrrolidone (PVP). (B) Effect of viscosity on swimming speed of WT and glycosylation-defective mutants from *P. syringae* pv. *tabaci* 6605.

FIG. 3. Polymorphic transitions of flagella filaments from WT and glycosylation-defective mutants from *P. syringae* pv. *tabaci* 6605. (A) Dark field light micrographs of flagella. Typical images of Coiled, Semi-Coiled, and a mixture of Semi-Coiled and Normal filaments prepared from WT and undulant filaments prepared from Δ*fgt1* and 6 S/A mutants strains. (B) Phase diagrams of polymorphs by pH and NaCl concentration. (SC: Semi-Coiled, No: Normal, Co: Coiled, St: Straight, DP: depolymerized).

FIG. 4. Bundle formation of flagella from glycosylation-defective mutants. (A) Bundle formation of flagella in swimming bacteria (WT, Δ*fgt1* and 6 S/A mutants) under a dark field microscope. (B) Electron micrographs of each strain. Insets are magnifications of the entangled flagella.

Fig. 1
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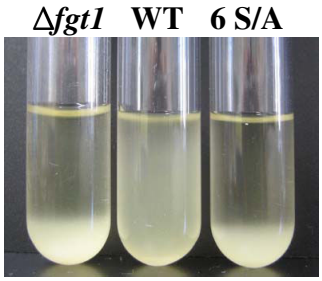

Fig. 2
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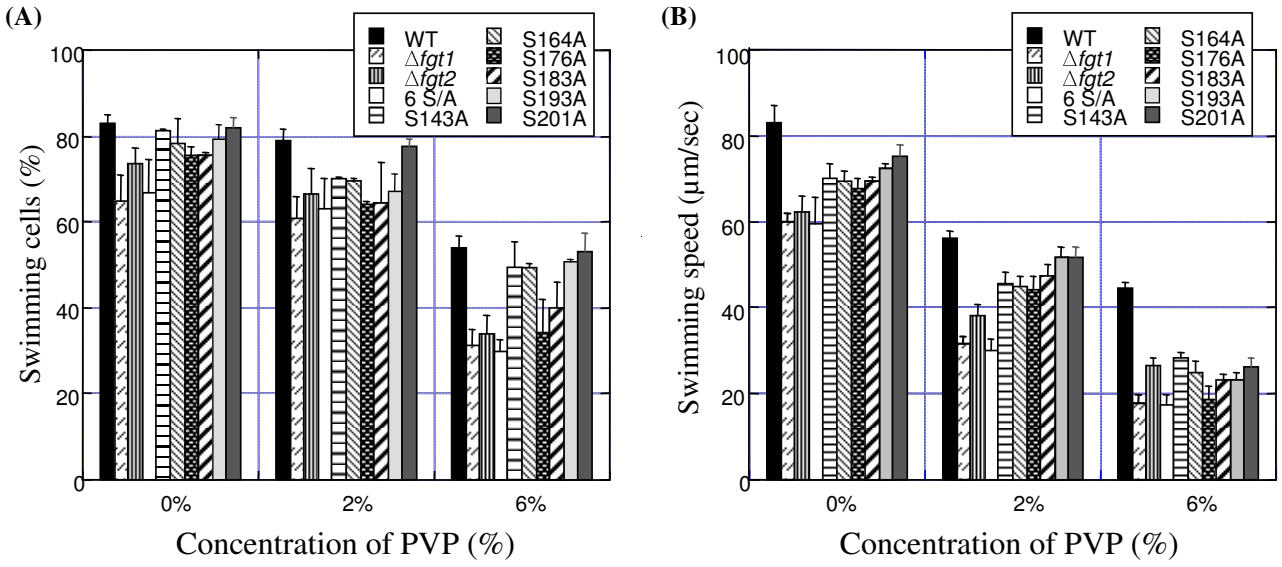

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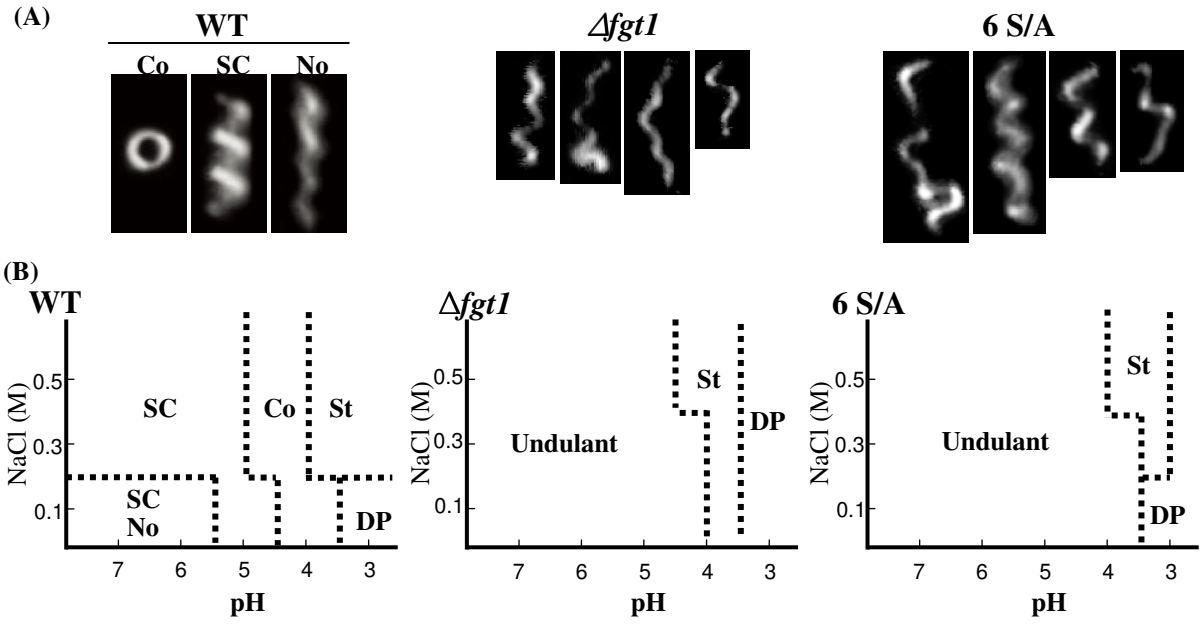

Fig. 4
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