

Defects in flagellin glycosylation affect the virulence of *Pseudomonas syringae* pv. *tabaci* 6605

Fumiko Taguchi,¹ Masanobu Yamamoto,² Mayumi Ohnishi-Kameyama,² Masako Iwaki,¹ Mitsuru Yoshida,² Tadashi Ishii,³ Tomoyuki Konishi³ and Yuki Ichinose¹

Correspondence

Yuki Ichinose

yuki@cc.okayama-u.ac.jp

¹Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530 Japan

²National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

³Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan

Flagellar motility and its glycosylation are indispensable for the virulence of *Pseudomonas syringae* pv. *tabaci* 6605. Six serine residues of the flagellin protein at positions 143, 164, 176, 183, 193 and 201 are glycosylated, and the glycan structure at 201 was determined to consist of a trisaccharide of two L-rhamnosyl residues and a modified 4-amino-4,6-dideoxyglucosyl (viosamine) residue. To investigate the glycan structures attached to the other serine residues and to identify the glycans important for virulence, Ser/Ala-substituted mutants were generated. Six mutant strains that each retained a single glycosylated serine residue were generated by replacing five of the six serine residues with alanine residues. MALDI-TOF mass analysis of flagellin proteins revealed that the major component of each glycan was a trisaccharide basically similar to that at position 201, but with heterogeneity in glycoform distribution. Swarming motility and amounts of acylhomoserine lactones (AHLs) as quorum-sensing signal molecules were significantly reduced, especially in the S143-5S/A, S164-5S/A and S201-5S/A mutants, whereas tolerance to antibiotics was increased in these three mutants. All the mutants showed lower ability to cause disease on host tobacco plants. These results supported our previous finding that glycosylation of the most externally located sites on the surface of the flagellin molecule, such as S176 and S183, is required for virulence in *P. syringae* pv. *tabaci* 6605. Furthermore, it is speculated that flagellum-dependent motility might be correlated with quorum sensing and antibiotic resistance.

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INTRODUCTION

Pseudomonas syringae pv. *tabaci* 6605 is a Gram-negative phytopathogenic bacterium and the causal agent of wildfire disease on tobacco plants (Ichinose *et al.*, 2003; Taguchi *et al.*, 2006b). This bacterium has several flagella at the cell pole. Previous results in our laboratory revealed that flagella are indispensable for virulence in host tobacco infection, and that a major component of flagellum, the flagellin protein, which is a *fliC* gene product, acts as the potential elicitor of the defence response in plants (Ichinose *et al.*, 2003; Shimizu *et al.*, 2003; Taguchi *et al.*, 2003a, b, 2006b). It was also revealed that glycosylation of flagellin is catalysed by two glycosyltransferases named FGT1 and FGT2, whose genes are located in a flagellin glycosylation island, and that $\Delta fgt1$ and $\Delta fgt2$ mutants produced non-glycosylated and

partially glycosylated flagellins, respectively (Taguchi *et al.*, 2006a; Takeuchi *et al.*, 2003, 2007). The HPLC analysis of digested fragments of flagellin protein suggested that the six serine residues at positions 143, 164, 176, 183, 193 and 201 are glycosylated (Taguchi *et al.*, 2006b). Characterization by MALDI-TOF mass analysis of each site-replaced Ser/Ala mutant confirmed that the molecular mass of the glycan at each glycosylated position was about 540 Da. The six Ser/Ala-substituted (S143A, S164A, S176A, S183A, S193A, and S201A) mutants had lower ability to adhere to hydrophobic surfaces, lower swarming motility, and lower virulence on the host tobacco plant, indicating that glycosylation of flagellin in this bacterium is required for virulence (Taguchi *et al.*, 2006b). Among the single Ser/Ala-substituted mutants, the effect of the Ala substitution in S176A and S183A was strongest, suggesting that the glycosylated positions on the putative surface-exposed region are important determinants of flagella-mediated virulence (Taguchi *et al.*, 2006b, 2008).

Abbreviations: EPS, extracellular polysaccharide; HHL, *N*-hexanoyl-L-homoserine lactone; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone.

A supplementary figure is available with the online version of this paper.

The glycan structure at serine 201 of flagellin proteins from *P. syringae* pv. *tabaci* 6605 and *P. syringae* pv. *glycinea* race 4 has been identified (Takeuchi *et al.*, 2007). Analysis of the sugar composition, mass spectrometry and NMR spectroscopy revealed that a trisaccharide composed of two rhamnosyl (Rha) residues and one modified 4-amino-4,6-dideoxyglucosyl (viosamine) residue was attached to serine 201 of both pathovars. Interestingly, the glycans of the flagellin from *P. syringae* pv. *tabaci* 6605 possess only L-rhamnose (L-Rha), whereas the *P. syringae* pv. *glycinea* race 4 glycan contains also D-Rha at a ratio of 4:1 (Takeuchi *et al.*, 2007). Because the deduced amino acid sequences of flagellin from these pathovars are identical, post-translational modification of glycosyl residues, especially in the different ratios of D and L configurations, might be important for the virulence and determination of host specificity.

In this study, to elucidate the glycan structures attached to other glycosylation sites and to determine the most important position for virulence, a series of six Ser/Ala-substituted mutants (S143-5S/A, S164-5S/A, S176-5S/A, S183-5S/A, S193-5S/A and S201-5S/A) was generated in *P. syringae* pv. *tabaci* 6605 by site-directed mutagenesis. Each mutant strain retained one of the six glycosylated serine residues. Using these glycosylation-defective mutant strains, detailed MS analysis was used to identify the glycan structure attached to each glycosylation site, and its correlation with determinants of flagellar motility and virulence, such as the production of *N*-acylhomoserine lactones (AHLs) and antibiotic resistance, was investigated.

METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. *Pseudomonas syringae* pv. *tabaci* 6605 strains were maintained as described previously (Taguchi *et al.*, 2006b). *Escherichia coli* strains were grown at 37 °C in Luria–Bertani (LB) medium. *Chromobacterium violaceum* CV026 was maintained at 30 °C in LB with kanamycin at a final concentration of 50 µg ml⁻¹ (McClellan *et al.*, 1997). The culture volume of each strain was 3 ml, and shaking conditions were 130 r.p.m. for *E. coli* and 110 r.p.m. for *P. syringae* pv. *tabaci* 6605.

Plant material, inoculation procedure, and bacterial growth. Tobacco plants (*Nicotiana tabacum* ‘Xanthi NC’) were grown at 25 °C with a 12 h photoperiod. For dip-inoculation, bacterial strains were suspended in 10 mM MgSO₄ and 0.02% Silwet L77 (OSI Specialties) at a density of 6 × 10⁸ c.f.u. ml⁻¹, and detached tobacco leaves (three leaves for each strain) were soaked in the suspension for 20 min. The inoculated leaves were incubated under conditions of 85% humidity in a growth cabinet for 10 days at 23 °C with an 18 h photoperiod. To maintain the turgidity of the detached leaves, water was supplied to the cut petiole.

To examine the bacterial growth, five leaf discs (5 mm diameter) were punched from tobacco leaves and soaked for 20 min in a suspension of bacteria in 10 mM MgSO₄ and 0.02% Silwet at a density of 6 × 10⁸ c.f.u. ml⁻¹. At 3 or 6 days after inoculation, the discs were soaked in 15% H₂O₂ for 1 min to sterilize the leaf surface, washed with sterile distilled water, and ground in a mortar. Serially diluted samples (10 µl) in 10 mM MgSO₄ were spotted on KB plates; the numbers of colonies that appeared after 48 h incubation at 27 °C were counted, and the bacterial populations were calculated.

Construction of mutants. To construct the six glycosylation-defective mutants, a Quik Change XL site-directed mutagenesis kit was employed as described previously (Taguchi *et al.*, 2006b). Each

Table 1. Bacterial strains and plasmids

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

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
DH5α	F ⁻ λ ⁻ ø80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r _K ⁻ m _K ⁻) supE44 thi-1 gyrA relA1	Takara
S17-1	thi pro hsdR hsdR hsdM ⁺ recA(chr::RP4-2-Tc::Mu-Km::Tn7)	Schäfer <i>et al.</i> (1994)
<i>P. syringae</i> pv. <i>tabaci</i>		
Isolate 6605	Wild-type isolated from tobacco, Nal ^r	Shimizu <i>et al.</i> (2003)
6605-dC	Isolate 6605 ΔfliC, Nal ^r	Shimizu <i>et al.</i> (2003)
6605-d1	Isolate 6605 Δfgt1, Nal ^r	Taguchi <i>et al.</i> (2006b)
6605-S143-5S/A	Isolate 6605 S164A, S176A, S183A, S193A, S201A Nal ^r	This study
6605-S164-5S/A	Isolate 6605 S143A, S176A, S183A, S193A, S201A Nal ^r	This study
6605-S176-5S/A	Isolate 6605 S143A, S164A, S183A, S193A, S201A Nal ^r	This study
6605-S183-5S/A	Isolate 6605 S143A, S164A, S176A, S193A, S201A Nal ^r	This study
6605-S193-5S/A	Isolate 6605 S143A, S164A, S176A, S183A, S201A Nal ^r	This study
6605-S201-5S/A	Isolate 6605 S143A, S164A, S176A, S183A, S193A Nal ^r	This study
6605-dpsyl	Isolate 6605 Δpsyl, Nal ^r	Taguchi <i>et al.</i> (2006b)
<i>C. violaceum</i>		
CV026	Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532; AHL biosensor	McClellan <i>et al.</i> (1997)
Plasmids		
pGEM-TEasy	3.015 kb cloning vector for PCR product; Amp ^r	Promega
pK18mobSacB	Small mobilizable vector, Km ^r , sucrose sensitive (<i>sacB</i>)	Schäfer <i>et al.</i> (1994)

mutation was confirmed by sequencing, and mutated DNA fragments were cloned into the mobilizable cloning vector pK18mobsacB via a *Bam*HI site (Schäfer *et al.*, 1994). The resulting plasmids were introduced into *E. coli* S17-1 and integrated into the wild-type (WT) *P. syringae* pv. *tabaci* 6605 by conjugation and homologous recombination. Nucleotide sequences of each mutant strain were confirmed by direct sequencing of bacterial genomic DNA by PCR.

Motility assay. Bacteria cultured overnight in LB medium containing 10 mM MgCl₂ at 25 °C were resuspended in 10 mM MgSO₄ and adjusted to an OD₆₀₀ of 0.3. Aliquots (3 µl) were inoculated into the centre of MMMF (50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, and 1.7 mM NaCl, pH 5.7, containing 10 mM of mannitol and fructose) plates with 0.25 % agar for the swimming assay (Taguchi *et al.*, 2006b) or SWM (0.5 % peptone, 0.3 % yeast extract; Difco) plates with 0.45 % agar for the swarming assay (Kinscherf & Willis, 1999). The diameters of the swimming or swarming areas were compared after incubation for 48 h at 25 °C or for 24 h at 27 °C, respectively.

Tolerance to antibiotics. Each strain was grown for 24 h in KB liquid medium, and 3 ml bacterial suspension was overlaid after dilution with 15 ml KB medium with 0.5 % agar. A paper disc (Whatman 3MM paper) containing 10 µl of antibiotic solution (chloramphenicol at 17.5 µg µl⁻¹ and spectinomycin at 25 µg µl⁻¹) was put on the bacteria-containing plate. The diameter of the growth inhibition zone was measured after incubation for 24 h at 27 °C (Taguchi *et al.*, 2006a).

Drop-collapsing test for biosurfactant detection. Each bacterial strain was grown on a KB plate containing 1.5 % agar for 48 h at 27 °C, scraped off, and suspended in 10 mM MgSO₄. After adjustment to an OD₆₀₀ of 1.0, 10 µl aliquots of the supernatant were spotted onto Parafilm to determine the surface tension (Taguchi *et al.*, 2006b).

Quantitative analysis of extracellular polysaccharide (EPS). Each bacterial strain was grown on a KB plate containing 1.5 % agar for 48 h at 27 °C. The detailed procedure was described in previous papers (Keith & Bender, 1999; Taguchi *et al.*, 2006b). Quantification of EPS was carried out by the phenol/sulfate method (Hodge & Hofreiter, 1962). The protein concentration of each sample was determined by a Bradford protein assay (Bio-Rad protein assay). Total EPS was calculated as a relative value per total cellular protein.

Detection of acylhomoserine lactones (AHLs). Bacterial strains were grown in LB medium with 10 mM MgCl₂ for 24 h at 25 °C. AHLs were extracted with an equal volume of ethyl acetate (Shaw *et al.*, 1997; Taguchi *et al.*, 2006a). The 500-fold-concentrated samples were applied to a C₁₈ reverse-phase TLC plate, and AHLs were detected with *C. violaceum* CV026 as the biosensor strain. For quantification of AHLs, the area of violet spots visualized by the induction of violacein production was calculated using *N*-hexanoyl-L-homoserine lactone (HHL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) as the standards.

Flagellin preparation. The procedure for flagellin purification from the WT and each mutant strain has been described (Taguchi *et al.*, 2006b).

RNA preparation and semiquantitative RT-PCR. Each strain was grown in LB with 10 mM MgCl₂ at 27 °C to an OD₆₀₀ of 0.3 and further incubated in MMMF for 1 h. Then total RNA was extracted using a High Pure RNA Isolation kit (Roche) according to the manufacturer's instructions. Two micrograms of total RNA was used for semiquantitative RT-PCR analysis (Higashi *et al.*, 2008). Specific primer sets, MexF-F (5'-TACAGCGAGCCAGATAAAC-3') and

MexF-R (5'-GAAGCGTACATGGCGATCTT-3'), ToIC-F (5'-CGTT-TGTCGTAATCGCTGAA-3') and ToIC-R (5'-AAACTGCCTGCT-ATCGCCAA-3'), were designed based on the registered sequences of *P. syringae* pv. *phaseolicola* 1448A (accession number NC_005773). PCR was performed with one denaturation cycle of 2 min at 95 °C and 32 cycles of 30 s at 95 °C, 30 s at 52 °C, and 40 s at 72 °C. Ten microlitres of the PCR product was loaded onto 2 % agarose gel. To confirm the mRNA-derived PCR amplification, the RT-PCR was performed without AMV RTase as a negative control.

Mass spectrometry of flagellin proteins. Each purified flagellin from the WT and S/A-substituted mutants was digested with trypsin (proteomics grade, Sigma-Aldrich) at 37 °C for 18 h in 10 mM Tris/HCl buffer (pH 8.0). The peptide mixture solution obtained was subjected to solid-phase extraction (ZipTip C4 pipette tips, Millipore) and was eluted with 50 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) after washing with MilliQ water (Millipore). The solution was mixed with a matrix solution (10 mg ml⁻¹) of sinapinic acid (Bruker Daltonics) in 30 % acetonitrile in 0.1 % TFA or 2,5-dihydroxybenzoic acid (Bruker Daltonics) in 20 % ethanol, followed by application of the mixture to a MALDI-TOF mass target plate. MALDI-TOF mass spectra were recorded on a Reflex II instrument (Bruker Daltonik) in the positive-ion mode by summing at least 100 shots of a 337 nm nitrogen laser with an acceleration voltage of 20 kV. The mass spectrometer was calibrated prior to measurements using commercially available standard proteins (a mixture of insulin, ubiquitin I, cytochrome *c* and myoglobin; Bruker Daltonics) covering the 5000 to 17 500 *m/z* range.

Statistical analysis. The results of the motility assay, EPS assay, TLC assay for AHLs, bacterial population measurement, and growth inhibition assay with antibiotics are expressed as means ± SD. Comparisons between the quantitative measurements of the WT and each mutant were performed by the two-tailed *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Construction of site-directed glycosylation-defective mutants

Previously, we confirmed the presence of glycosylated residues in flagellin (positions 143, 164, 176, 183, 193 and 201) and the structure of the glycan at serine 201 (S201) was determined (Taguchi *et al.*, 2006b, 2007). To analyse the structure of the flagellin glycans attached to the other five glycosylated residues, a series of six Ser/Ala-substituted mutants (S143-5S/A, S164-5S/A, S176-5S/A, S183-5S/A, S193-5S/A and S201-5S/A) was generated using site-directed mutagenesis, conjugation and homologous recombination. The mutations were confirmed by DNA sequencing, and the reduction of the molecular mass of flagellin from all glycosylation defective mutants was confirmed by SDS-PAGE (Fig. 1A).

Mass spectrometry of flagellin from glycosylation-defective mutants

Mass differences between the WT and each of the five Ser/Ala-substituted mutant flagellins were analysed more precisely by MALDI-TOF mass spectrometry after tryptic digestion. Enzymic hydrolysis of the WT flagellin gave a

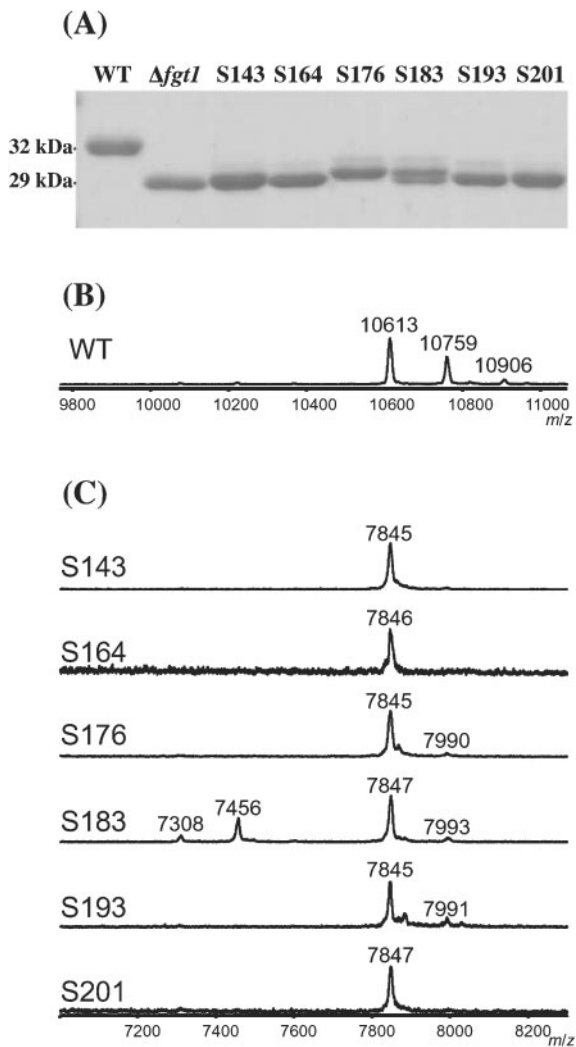


Fig. 1. SDS-PAGE (A) and MALDI-TOF mass spectra of tryptic digested peptides (136N–210R) of flagellin proteins from the WT (B) and glycosylation-defective mutants (C). $\Delta fgt1$, S143, S164, S176, S183, S193 and S201 indicate the $\Delta fgt1$, S143-5S/A, S164-5S/A, S176-5S/A, S183-5S/A, S193-5S/A and S201-5S/A (S201) mutants, respectively. Peaks observed around the $[M+H]^+$ peak were $[M+Na]^+$ and $[M+K]^+$.

glycopeptide 136N–210R including all six glycosylated serine residues. The protonated ion $[M+H]^+$ was observed at m/z 10 610 in good agreement with the theoretical value of 10 613.4 for the peptide 136N–210R (7387.0) with six flagellin glycan chains (6×537.5) (Fig. 1B). Here, the flagellin glycan chain was regarded as a trisaccharide consisting of two rhamnose residues and one modified viosamine residue [β -D-Quip4N(3-OH-1-oxo-Bu)2Me], which was identified as the glycan on 201S (Takeuchi *et al.*, 2007). The other two peaks observed in the higher mass region corresponded to glycopeptides including one and two more rhamnose residues because the mass differences from $[M+H]^+$ at m/z 10 613 were 146 and

293, respectively. In contrast to the WT flagellin, the series of five Ser/Ala-substituted mutant flagellins showed protonated ions at around m/z 7845 (Fig. 1C). This value corresponded to the total molecular masses of the five Ser/Ala-substituted peptides without glycan modifications (7308.0 Da) and the glycan chain (537.5 Da). The results revealed that all five Ser/Ala-substituted mutant flagellins each had only one flagellin glycan of 537–539 Da, and that the glycan mass was similar to that of serine 201, 538 Da. However these molecular masses showed some heterogeneity. We detected minor peaks, especially in the S183-5S/A mutant. The peaks of the S183-5S/A mutant observed at m/z 7308 and m/z 7456 indicate the molecular masses of flagellin protein without post-translational modification and with attachment of only a single rhamnose residue, respectively. The profiles of S176-5S/A, S183-5S/A and S193-5S/A each showed a minor peak around m/z 7990, suggesting the existence of an additional rhamnose residue in the peptide. Uniform glycosylation might be necessary for accurate attachment of rhamnose residues by glycosyltransferases.

Motility of glycosylation-defective mutants

The swimming and swarming motilities of each glycosylation-defective mutant were compared with those in the WT strain on 0.25 % agar MMMF plates and 0.45 % agar SWM plates, respectively. There was a small but significant difference in swimming motility among the WT and mutant strains (Fig. 2A). All mutant strains also showed reduced swarming motility (Fig. 2B). In particular, the S143-5S/A, S164-5S/A and S201-5S/A mutants were markedly impaired in swarming ability, but the S176-5S/A, S183-5S/A and S193-5S/A mutants retained significant swarming motility. Because the glycosylation positions at S176 and S183 are located on the most external surface (Taguchi *et al.*, 2006b) according to the predicted tertiary structure of flagellin, removal of glycans in these positions might have a greater effect than removal of those in other positions. This result was consistent with our previous finding that a single S176A- or S183A-substituted mutant exhibited reduced swarming motility compared with other single Ser/Ala mutants (Taguchi *et al.*, 2006b). Quantitative analyses of swimming and swarming motilities are summarized in Table 2.

Drop-collapsing test

The production of biosurfactant in WT and mutant strains was investigated by the drop-collapsing test. The results indicated that there was no significant difference among the strains tested (see Supplementary Fig. S1, available with the online version of this paper).

Detection of AHLs by TLC

Quorum sensing regulates bacterial virulence factors in a cell-density-dependent manner. We previously reported

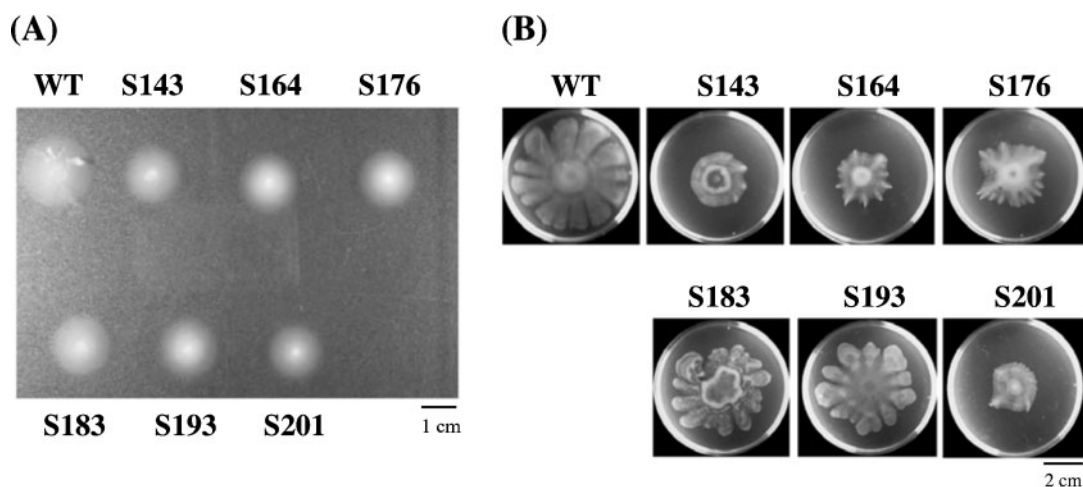


Fig. 2. Swimming (A) and swarming (B) assays. Aliquots (3 μ l) of each bacterial suspension (6×10^8 c.f.u. ml $^{-1}$) were inoculated on 0.25 % agar MMMF plates for swimming assay or 0.45 % agar SWM plates for swarming assay. Each plate was incubated for 48 h at 27 °C. The photographs show representative results obtained from three independent experiments.

that *P. syringae* pv. *tabaci* 6605 produces HHL and OHHL as two major quorum-sensing signal molecules (Taguchi *et al.*, 2006a). We also found that the flagellin-defective Δ *fliC* mutant is incapable of producing AHLs (Fig. 3). This result suggests that flagellar motility might be necessary for optimal production of AHLs. Therefore, TLC using *C. violaceum* CV026 as the AHL biosensor strain was performed to compare the ability of each glycosylation-defective mutant to produce AHLs. As shown in Fig. 3, the AHL production by the S176-5S/A, S183-5S/A and S193-5S/A mutants was almost identical to that in the WT strain. In contrast, only a small amount of AHL was produced by the S143-5S/A and S201-5S/A mutants and Δ *fgt1*, a non-glycosylated mutant. This result may indicate a close relationship between AHL production and swarming motility. The quantitative analysis of AHL production is summarized in Table 2.

Tolerance to antibiotics

We previously reported that quorum sensing negatively regulated the production of EPS, and that AHL-deficient mutants (Δ *psyI* and Δ *orf3*) were more resistant to antibiotic treatment (Taguchi *et al.*, 2006a). Because some flagellin glycosylation-defective mutants have decreased ability to produce AHLs (Fig. 3), a growth inhibition test using chloramphenicol and spectinomycin was performed to compare susceptibility to these antibiotics. As shown in Fig. 4, Δ *fliC* and Δ *fgt1* mutants and the AHL-defective Δ *psyI* mutant had markedly increased tolerance to these antibiotics. The S176-5S/A, S183-5S/A and S193-5S/A mutants showed slightly increased antibiotic tolerance in the growth inhibition assay, whereas the S143-5S/A, S164-5S/A and S201-5S/A mutants showed higher tolerance to these antibiotics. These results suggest a possible correlation among quorum sensing via AHL production, swarming motility, and susceptibility to antibiotics.

Table 2. Quantitative analysis of motility and production of EPS and AHLs

Data shown are the means \pm SD of three independent replicates. Statistical significance is indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

Strains	Motility		EPS [A_{490} (mg protein) $^{-1}$]	AHLs [ng (ml medium) $^{-1}$]	
	Swim (cm)	Swarm (cm)		HHL	OHHL
WT	2.39 \pm 0.063	6.67 \pm 0.15	0.905 \pm 0.187	97.6 \pm 12.6	76.4 \pm 2.11
143S-5S/A	1.83 \pm 0.050**	1.80 \pm 0.20**	0.701 \pm 0.239	17.3 \pm 0.95**	11.0 \pm 2.07**
164S-5S/A	1.85 \pm 0.100*	1.80 \pm 0.12**	0.567 \pm 0.045*	16.7 \pm 2.40**	11.2 \pm 2.63**
176S-5S/A	1.88 \pm 0.130*	3.92 \pm 0.25**	0.646 \pm 0.278	48.0 \pm 5.55*	39.6 \pm 2.93**
183S-5S/A	1.86 \pm 0.110*	5.37 \pm 0.11**	0.739 \pm 0.297	76.5 \pm 5.71	37.5 \pm 2.93**
193S-5S/A	1.85 \pm 0.129*	5.07 \pm 0.11**	0.584 \pm 0.097	73.2 \pm 9.63	39.9 \pm 3.96*
201S-5S/A	1.83 \pm 0.043**	1.83 \pm 0.25**	0.627 \pm 0.178	9.76 \pm 2.40**	8.76 \pm 0.54**

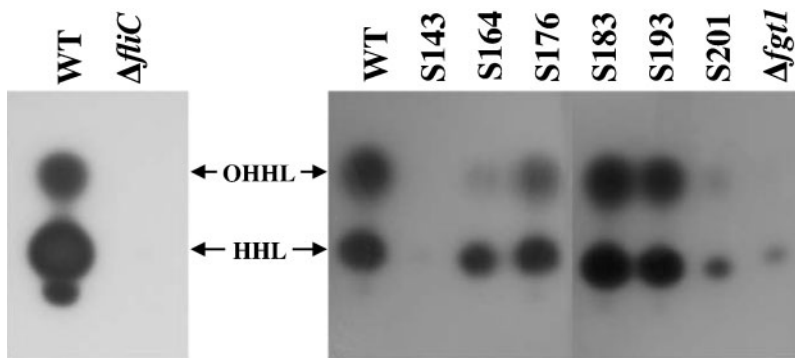


Fig. 3. Detection of AHLs using *C. violaceum* CV026 as biosensor strain. Ethyl acetate extracts from each strain were developed on a C_{18} reverse-phase TLC plate. The photographs show representative results obtained from three independent experiments.

EPS production

The production of EPS was investigated in WT and mutant strains (Table 2). No significant difference was found in the amounts of EPS produced by the WT and glycosylation-defective mutant strains, except mutant S164-5S/A.

Virulence of mutants on tobacco leaves

To compare the abilities of the WT and each glycosylation-defective mutant to cause disease on tobacco leaves, dip-inoculation of each bacterium at 6×10^8 c.f.u. ml^{-1} was carried out. The results showed that virulence to host tobacco plants was significantly attenuated in all of the mutants (Fig. 5A). The leaves inoculated with the S176-5S/A and S183-5S/A mutants exhibited slight chlorosis, suggesting that these mutants retain some virulence. The populations of the WT strain and each mutant were examined 3 and 6 days after inoculation. As shown in Fig. 5(B), all of the mutants had less growth than the WT strain, especially mutants S143-5S/A, S164-5S/A and S201-5S/A.

Expression of genes related to drug resistance

Because antibiotic tolerance is related to active multidrug efflux pumps, expression of two genes related to drug resistance was investigated by RT-PCR (Fig. 6). Among the active drug efflux pumps, transporters of the resistance-nodulation-division family (RND superfamily) have a predominant role in Gram-negative bacteria (Nikaido, 2009). The RND multidrug efflux pumps are associated with two other classes of proteins, outer-membrane proteins such as TolC in *E. coli* and membrane fusion proteins such as AcrA in *E. coli*. Significant expression of the genes encoding MexF, a member of the RND pump family, and TolC were observed in $\Delta fliC$, $\Delta fgt1$ and $\Delta psyI$ mutant strains but expression was not detected in the WT strain. The *mexF* and *tolC* genes belong to the same operon in *P. aeruginosa* and *P. syringae*.

DISCUSSION

Previously, we determined the glycan structure of flagellin protein at serine 201 using a digested peptide derived from

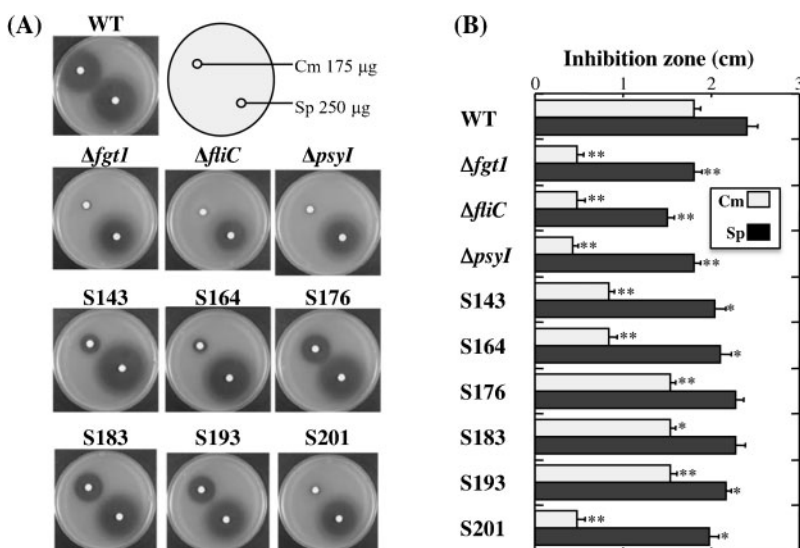


Fig. 4. Antibiotic tolerance tests. (A) Paper discs containing 10 μl of antibiotic solution (17.5 μg chloramphenicol μl^{-1} and 25 μg spectinomycin μl^{-1}) were placed on each bacterium-containing KB plate. Photographs were taken after 24 h at 27 $^{\circ}C$. (B) The diameter of the growth-inhibition zone was measured after incubation for 24 h at 27 $^{\circ}C$. Data shown are the means \pm SD of three independent experiments. Asterisks indicate a significant difference from the the WT in a *t*-test (* $P < 0.05$, ** $P < 0.01$).

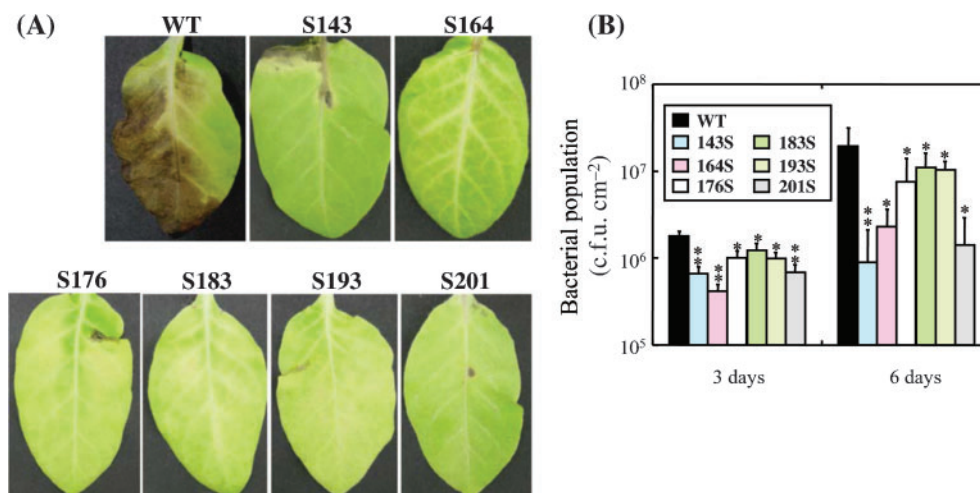


Fig. 5. Virulence assays. (A) Tobacco leaves were dip-inoculated with each bacterium at 6×10^8 c.f.u. ml⁻¹ for 20 min and incubated at 23 °C. Photographs were taken 10 days after inoculation. Representative results from three independent experiments are shown. (B) Bacterial growth in tobacco leaf discs was calculated at 3 and 6 days post-inoculation. The bars represent means \pm SD for three experiments. Asterisks indicate a significant difference from the WT in a *t*-test (* $P < 0.05$, ** $P < 0.01$).

flagellin of the WT strain (Takeuchi *et al.*, 2007), by means of sugar composition analysis, mass spectrometry, and ¹H and ¹³C NMR spectroscopy. Digestion of flagellin by Asp-N endoproteinase generated three fractions (D200–A211, D168–T187 and D139–F167) and the D200–A211 fraction had a single glycosylation site at S201; therefore, we succeeded in the structural analysis of this glycan. However, we had no suitable peptidase to target peptides containing other single glycosylation sites for detailed structural analysis. In this study, site-directed Ser/Ala substitutions were constructed: in each, a single glycosylated serine remained, but Ser was replaced by an Ala residue in the other five glycosylated sites to obtain mutant flagellins

that possessed only a single glycan. Flagellin proteins prepared from these mutants are convenient tools for structural analysis of glycans. Although we did not generate complemented strains for each mutant, the multiple mutant strains showed similar phenotypes. Therefore, spontaneous mutations probably did not occur during the mutagenesis experiment, but this possibility can not be excluded.

Swarming motility has been reported to be linked to bacterial virulence and is enhanced by addition of biosurfactants, which are secreted by several bacteria to reduce surface tension (Brint & Ohman, 1995; Taguchi *et al.*, 2006b). Although the S143-5S/A, S164-5S/A and S201-5S/A mutants had reduced swarming ability, the production of the biosurfactant by these mutants showed no significant difference compared with the WT strain as assayed by the drop-collapsing test (Supplementary Fig. S1). We previously demonstrated that flagella from glycosylation-defective mutants ($\Delta fgt1$ and all six glycosylated Ser/Ala-substituted mutants) displayed irregular entanglements of filaments and many thick flagellar bundles (Taguchi *et al.*, 2008). Because flagella from glycosylation-defective mutants might have a different surface charge that reduces the hydrophilic properties, the swarming motility would be impaired. Flagellin glycosylation, especially at positions S176 and S183, contributed to smooth motility, and it was confirmed that glycosylation of the most surface-exposed positions plays an indispensable role in the swarming motility of *P. syringae* pv. *tabaci* 6605.

Many studies have shown that quorum sensing plays an important role in regulation of the expression of many virulence factors in Gram-negative bacteria (Daniels *et al.*, 2004). Our previous study demonstrated that *orf3* located

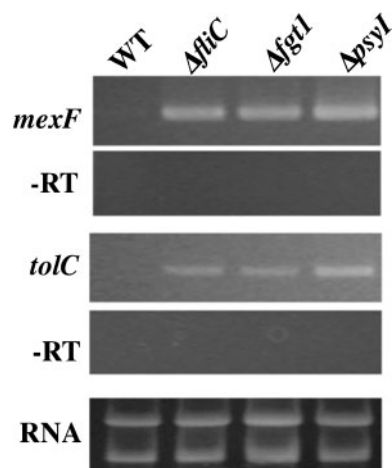


Fig. 6. Expression of *mexF* and *tolC* genes in each strain.

between *fgt2* and *fliC* plays no role in post-translational modification, but a $\Delta orf3$ mutant nearly lost the ability to produce AHLs, indicating that Orf3 is involved in production of AHLs as quorum-sensing signal molecules (Taguchi *et al.*, 2006b). The *orf3* product is highly homologous to the 3-oxoacyl-(acyl carrier protein) synthase III of *E. coli* and *Pseudomonas putida* strain KT2440 in the fatty acid elongation cycle (Nelson *et al.*, 2002; Takeuchi *et al.*, 2003). Although the reason why a gene concerned with quorum sensing is present in the glycosylation island of the flagellar gene cluster is still obscure, quorum sensing via Orf3 should be correlated with flagellum expression. As shown in Fig. 3, the amounts of AHL produced by the $\Delta fgt1$, S143-5S/A and S201-5S/A mutants were significantly reduced, and these mutants showed lower swarming ability. We observed that swimming- and swarming-defective mutants, such as $\Delta fliC$, had lost the ability to produce AHLs (Fig. 3). This result led us to speculate on a possible relationship between the synthesis of AHLs and flagellum-dependent swarming motility. To elucidate this hypothesis, we will investigate the relationship between the expression of genes required for flagellin glycosylation, such as *fliC* and *fgt1*, and genes required for AHL biosynthesis, such as *orf3* and *psyl*.

It was reported that a mutant of *P. syringae* deficient in EPS production had enhanced sensitivity to H₂O₂ (Keith & Bender, 1999; Daniels *et al.*, 2004; Quinones *et al.*, 2005), and the $\Delta orf3$ mutant of *P. syringae* pv. *tabaci* 6605 had enhanced tolerance to H₂O₂ and antibiotics due to the overexpression of EPS (Taguchi *et al.*, 2006a). In *Vibrio cholerae* O139 strain MO10, a relationship between the flagellum-dependent EPS signalling cascade and virulence was demonstrated by the result that disruption of the system of flagellar synthesis elevated EPS expression (Lauriano *et al.*, 2004). In *P. syringae* pv. *tabaci* 6605, although the S143-5S/A, S164-5S/A and S201-5S/A mutants were highly tolerant of antibiotics (Fig. 4), the amounts of EPS produced by these glycosylation-defective mutants, except S164-5S/A, were similar to that in the WT strain (Table 2). Therefore, the flagellum may affect tolerance to antibiotics and virulence via EPS-independent pathways in this bacterium.

The mechanisms of increased tolerance to antibiotics in the AHL production-defective mutants $\Delta fgt1$, $\Delta fliC$, $\Delta psyl$, S143-5S/A, S164-5S/A and S201-5S/A are not known. Two mechanisms for acquiring multidrug resistance in bacteria have been reported (Nikaido, 2009). One is the accumulation of multiple genes coding for resistance to drugs, and the second is increased expression of genes related to multidrug efflux pumps. We found that the expression of *mexF* and *tolC*, which are RND multidrug efflux pump related genes, was significantly elevated in motility-defective mutants and also in AHL synthesis-defective mutants (Fig. 6). In *P. aeruginosa*, mutation of genes encoding the efflux protein MexI and the porin OpmD resulted in the inability to produce OHHL and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and a marked reduction

in *N*-butanoyl-L-homoserine lactone levels (Aendekerk *et al.*, 2005). These mutants showed enhanced resistance to several classes of antibiotics compared with the WT strain. Aendekerk *et al.* (2005) further demonstrated that exogenous PQS restored AHL production as well as antibiotic susceptibility, indicating that the MexGHI-OpmD multidrug efflux pump is required for secretion of PQS and control of antibiotic susceptibility and virulence via PQS-dependent cell-to-cell communication. However, because OHHL was shown to be freely diffused through *Vibrio fischeri* cells (Kaplan & Greenberg, 1985), an active pump is not necessary for transport of this molecule. Although quorum-sensing molecules, including PQS, positively regulate antibiotic resistance in *P. aeruginosa*, an AHL-dependent quorum-sensing system might control antibiotic susceptibility by negative regulation of the *mexEF-tolC* gene cluster in *P. syringae* pv. *tabaci* 6605. Further characterization of these genes using respective mutant strains should elucidate the mechanism of flagellar motility-mediated quorum sensing and/or multidrug resistance in *P. syringae* pv. *tabaci* 6605. The findings of this study suggest the possibility of controlling virulence by using inhibitors of flagellum-dependent motility, making it possible to reduce the use of bactericides in agricultural fields.

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