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A Homologue of the 3-Oxoacyl-(Acyl Carrier Protein) Synthase III Gene Located in the Glycosylation Island of *Pseudomonas syringae* pv. tabaci Regulates Virulence Factors via *N*-Acyl Homoserine Lactone and Fatty Acid Synthesis▽

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***Pseudomonas syringae* pv. tabaci 6605 possesses a genetic region involved in flagellin glycosylation. This region is composed of three open reading frames: *orf1*, *orf2*, and *orf3*. Our previous study revealed that *orf1* and *orf2* encode glycosyltransferases; on the other hand, *orf3* has no role in posttranslational modification of flagellin. Although the function of Orf3 remained unclear, an *orf3* deletion mutant (Δ*orf3* mutant) had reduced virulence on tobacco plants. Orf3 shows significant homology to a 3-oxoacyl-(acyl carrier protein) synthase III in the fatty acid elongation cycle. The Δ*orf3* mutant had a significantly reduced ability to form acyl homoserine lactones (AHLs), which are quorum-sensing molecules, suggesting that Orf3 is required for AHL synthesis. In comparison with the wild-type strain, swarming motility, biosurfactant production, and tolerance to H_2O_2 and antibiotics were enhanced in the Δ*orf3* mutant. A scanning electron micrograph of inoculated bacteria on the tobacco leaf surface revealed that there is little extracellular polymeric substance matrix surrounding the cells in the Δ*orf3* mutant. The phenotypes of the Δ*orf3* mutant and an AHL synthesis (Δ*psyI*) mutant were similar, although the mutant-specific characteristics were more extreme in the Δ*orf3* mutant. The swarming motility of the Δ*orf3* mutant was greater than that of the Δ*psyI* mutant. This was attributed to the synergistic effects of the overproduction of biosurfactants and/or alternative fatty acid metabolism in the Δ*orf3* mutant. Furthermore, the amounts of iron and biosurfactant seem to be involved in biofilm development under quorum-sensing regulation in *P. syringae* pv. tabaci 6605.**

Pseudomonas syringae pv. tabaci 6605, an phytopathogenic bacterial isolate, causes wildfire disease on host tobacco plants and induces a hypersensitive reaction (HR) on nonhost plants. In previous studies, we demonstrated that flagellin, a component of the flagellar filament, is a major elicitor of HR by *P. syringae* pv. tabaci 6605 (30, 35). Flagellin of *P. syringae* pv. tabaci 6605 induces HR on nonhost plants but not on its host tobacco plant. Although the deduced amino acid sequence of *P. syringae* pv. glycinea race 4 flagellin (FliC) is identical to that of *P. syringae* pv. tabaci 6605 flagellin, the flagellin of *P. syringae* pv. glycinea does induce HR on tobacco plants, suggesting that posttranslational modification of flagellin determines the specificity to induce HR (34). We recently reported that genes existing upstream of *fliC*, which encodes flagellin protein, are involved in the glycosylation of flagellin in these two pathovars (33, 36). There are three open reading frames, namely, *orf1*, *orf2*, and *orf3*, in the glycosylation islands of *P. syringae* pv. tabaci and pv. glycinea (Fig. 1). Because the proteins encoded by *orf1* and *orf2* showed homology to a putative glycosyltransferase and the molecular mass of the flagellin of each *orf1* and *orf2* deletion mutant (Δ*orf1* and Δ*orf2* mutants) was decreased, these genes are thought to encode the glycosyltransferases. Inoculation of these mutants into each host plant confirmed that glycosylation of flagellin proteins plays an important role in their virulence (33, 36). While the Δ*orf3* mutants of *P. syringae* pv. tabaci also had significantly reduced virulence on host tobacco plants, mass spectrometry analysis indicated that *orf3* is not involved in posttranslational modification (33).

A homology search revealed that *orf3* is highly homologous to the putative 3-oxoacyl-(acyl carrier protein [ACP]) synthase III (β-ketoacyl-ACP synthase III) genes of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas putida* strain KT2440 (24, 36). It has been reported that a 3-oxoacyl-ACP synthase III called FabH is one of the enzymes in the type II fatty acid synthesis system (20). Most bacteria and plant plastids use this cycle, with each enzyme catalyzing an individual reaction to produce long-chain fatty acids. In this elongation cycle, 3-oxoacyl-ACP synthase III first catalyzes a condensation reaction to supply intermediates of short-chain fatty acids (14, 20).

Recently, the expression of many virulence factors has been reported to be regulated by a cell density-dependent system called quorum sensing. Several studies revealed that quorum sensing by gram-negative bacteria involves *N*-acyl homoserine lactones (AHLs) that differ in the structures of their *N*-linked acyl side chains as signal molecules (23, 29). AHLs are synthesized by the coupling of homoserine lactone rings from *S*-adenosylmethionine and acyl chains from the acyl-ACP pool in

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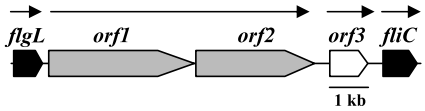

FIG. 1. Schematic organization of the glycosylation island in the flagellum gene cluster of *Pseudomonas syringae* pv. tabaci and pv. glycinea. *flgL*, gene encoding HAP3; *orf1* and *orf2*, genes encoding glycosyltransferases for flagellin glycosylation; *orf3*, putative gene encoding 3-oxoacyl-(acyl carrier protein) synthase III, *fliC*, gene encoding flagellin. Arrows indicate putative transcripts and directions of transcription.

cells by the enzymes LuxI in *Vibrio fischeri* (27) and PsyI in *P. syringae* (12). Because 3-oxoacyl-ACP synthase III correlates with fatty acid biosynthesis, we speculated that *orf3* has a critical role in AHL production. In addition, it was reported that other biosynthetic pathways, such as the synthesis of phospholipids and lipopolysaccharides, also use acyl-ACP intermediates (14), suggesting an important role for Orf3 in fatty acid cellular metabolism.

In this study, the role of the *orf3* gene in the glycosylation island of the flagellin gene cluster of *P. syringae* pv. tabaci 6605 was investigated by analysis of the AHL production and some virulence factors under regulation of quorum sensing in the Δ*orf3* mutant. In addition, we generated the AHL-defective Δ*psyI* mutant and compared its characteristics with those of the Δ*orf3* mutant. The physiological role of the *orf3* gene in biofilm formation on the tobacco leaf surface was also examined.

MATERIALS AND METHODS

Bacterial strains and growth condition. All bacterial strains used in this study are shown in Table 1. *Pseudomonas syringae* pv. tabaci 6605 strains were maintained as described previously (35). *E. coli* strains were grown at 37°C in Luria-Bertani medium (LB). *Chromobacterium violaceum* CV026 as the AHL biosensor strain was maintained at 30°C in LB with a final concentration of 50 μg/ml kanamycin (22).

Plant material and inoculation procedure. Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 25°C with a 12-h photoperiod. For the inoculation experiments, bacterial strains were suspended in 10 mM $MgSO_4$ and 0.02% Silwet L77 (OSI Specialties, Danbury, CT) at a density of 2×10^8 CFU/ml. After spray inoculation on both surfaces of tobacco leaves, the leaves were incubated in a growth cabinet for 8 days at 23°C.

Construction of mutants. Generation of the Δ*orf3* mutant and its complementary strain by pDSKGI (Table 1) from *P. syringae* pv. tabaci 6605 was described previously (33). To generate an AHL synthesis-defective (Δ*psyI*) mutant, the genetic region of *psyI* and *psyR* was first isolated by using a TA cloning system (pGEMT-Easy; Promega, Tokyo, Japan). PCR primers (PsyI5′ [5′-ATGTCGAGCGGGTTTGAGTTTCAG-3′] and PsyR5′ [5′-ATGGAGGTTCGTACCGTGAAAGCC3′]) were designed based on the registered sequences of *psyI* and *psyR* of *P. syringae* pv. tabaci (accession number AF110468). The Δ*psyI* mutant was constructed by the replacement of the codon AAG for Lys^{148} with a TAG stop codon by using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two complementary oligonucleotides containing a stop codon, PsyI-S (5′-CACGGTGGTCAGCTAGGCAATGGCGCGGAT-3′) and PsyI-AS (5′-ATCCGCGCCATTGCCTAGCTGACCACCGTG-3′), were synthesized. Mutation was introduced according to the manufacturer's protocol and confirmed by sequencing.

Detection of AHL by TLC analysis. Bacterial strains were grown in King's B (KB) medium for 24 h at 25°C. After removal of cells by centrifugation, AHLs were extracted from the supernatant with an equal volume of ethyl acetate, and the organic phases were evaporated. Residues were dissolved in 1/500 the original volume of ethyl acetate, and 10 μl of the solution was subjected to C_{18} reverse-phase thin layer chromatography (TLC) (20 cm by 20 cm, RP-18F254S; Merck, Darmstadt, Germany) with a solvent system of methanol and water (60:40, vol/vol). After development, the dried TLC plate was overlaid with 50 ml of semisolid LB agar medium containing 6 ml of an overnight culture of *C. violaceum* CV026. After 24 h of incubation at 30°C, AHL was visualized as violet spots by the induction of violacein production. Chemically synthesized *N*-hexanoyl-L-homoserine lactone (HHL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) as the standard molecules were gifts from T. Ikeda (Utsunomiya University). All samples were stored at −20°C in ethyl acetate.

Northern blot analysis. *Pseudomonas* strains were incubated in LB with 10 mM $MgCl_2$ for 24 h at 25°C. The cells were transferred to new minimal medium (MM) [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 (MMMF) (30) and incubated to an optical density at 600 nm (OD_{600}) of 0.3. Total RNA was extracted using a High Pure RNA isolation Kit (Roche, Mannheim, Germany), and 10 μg of RNA was used for Northern blot analysis. The probes were labeled with a PCR digoxigenin synthesis kit (Roche). The conditions for hybridization and detection were as described by Shimizu et al. (30).

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5a	F^- λ^- ϕ80d*lacZΔM15* Δ*(lacZYA-argF)U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	Takara, Kyoto, Japan
S17-1	*thi pro hsdR hsdM*$^+$ *recA* [*chr*::RP4-2-Tc::Mu-Km::Tn*7*]	26
Chromobacterium violaceum CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532; AHL biosenser	22
Pseudomonas syringae pv. tabaci		
6605	Wild type; Nal^r	30
6605-d3	6605 Δ*orf3*	33
6605-dpsyI	6605 Δ*psyI*	This study
Plasmids		
pGEM-T Easy	3.015-kb cloning vector for PCR product; Amp^r	Promega, Tokyo, Japan
pK18*mobsacB*	Small mobilizable vector; Km^r; sucrose sensitive (*sacB*)	26
pDSK519	Broad-host-range cloning vector; Km^r	16
pM3	1.69-kb chimeric PCR product deleting *orf3* cloned into pK18*mobsacB* at EcoRI site; Km^r	33
pDSKGI	9-kb HindIII fragment containing *orf1*, *orf2*, and *orf3* genes from *P. syringae* pv. tabaci 6605 in pDSK519; Km^r	33

[a] Amp^r, ampicillin resistance; Km^r, = kanamycin resistance; Nal^r, nalidixic acid resistance.

Scanning electron and optical microscopy. Leaves inoculated with the wild-type (WT) strain or mutant strains for 8 days at 23°C were observed by scanning electron microscopy. The detailed procedure has been described by Taguchi et al. (33). Colony morphologies of the WT and each mutant on 1.5% KB agar plates after 2 days of incubation were observed under an optical microscope (Olympus IX70, Tokyo, Japan).

Swarming assay. Bacteria cultured overnight in LB containing 10 mM $MgCl_2$ at 25°C were resuspended in 10 mM $MgSO_4$ and adjusted to an OD_{600} of 0.1. Three-microliter aliquots were inoculated in the center of the medium for swarming (SWM plate [0.5% peptone, 0.3% yeast extract, and 0.5% agar; Difco, Detroit, MI]) (18) or MMMF (MMMF plate with 0.5% agar) (33). The swarming motility was observed after 24 h of incubation at 27°C for SWM and at 23°C for MMMF agar plates.

Biosurfactant assay. Overnight cultures of WT and mutant strains in LB with 10 mM $MgCl_2$ were subcultured into MMMF and incubated for 24 h at 23°C. After centrifugation, aliquots of 10 μl of supernatant were spotted on the film (Parafilm; Alcan Packaging, Neenah, WI) to detect the drop-collapsing activity (21).

For biosurfactant detection, overnight cultures in LB containing 10 mM $MgCl_2$ at 25°C were centrifuged and adjusted to an OD_{600} of 0.1 with 10 mM $MgSO_4$. Five microliters of the bacterial suspension was placed on an MMMF agar plate with 0.0005% methylene blue and 0.02% hexadecyltrimetyl ammonium bromide, as described by Kohler et al. (19). The plate was incubated for 48 h at 27°C until the appearance of a blue halo.

Glycolipid detection by TLC. Each bacterial strain was grown in 200 ml of KB for 24 h at 25°C, and cells were removed by centrifugation. The supernatant was filtered through a 0.45-μm-pore-size filter, and glycolipids were extracted with an equal volume of ethyl acetate. The organic phase was evaporated to dryness and redissolved in 400 μl of ethyl acetate. Fifteen microliters of sample was subjected to TLC on a precoated silica gel glass plate (Silica Gel 60, Whatman Inc., Clifton, NJ) with a solvent system of chloroform-methanol-water (95:20:2, vol/vol/vol). After development, glycolipids were visualized by spraying with 0.2% orcinol reagent dissolved in 11.2% H_2SO_4 (final concentrations) and heating at 110°C for 15 min (31).

CAS assay. The siderophore production by bacterial strains in the culture supernatant was determined by the methods of Schwyn and Neilands (28). Each strain was incubated in LB containing 10 mM $MgCl_2$ for 24 h at 25°C and was adjusted to an OD_{600} of 0.001, 0.01, or 0.1 with MMMF. After 24 h of incubation, each culture was filtered through a 0.45-μm-pore-size filter (Millipore). Fifty microliters of each sample or MMMF medium as a reference was added to 150 μl of chromoazurol S (CAS) solution (0.6 mM hexadecyltrimetyl ammonium bromide, 0.015 mM $FeCl_3$, 0.15 mM HCl, and 0.15 mM CAS in MMMF). The absorbance of each sample at 630 nm was measured after 30 min of incubation at room temperature. The amount of siderophore was calculated by subtracting the absorbance value of the reference.

Tolerance to H_2O_2 and antibiotics. *P. syringae* pv. tabaci strains were grown for 24 h in KB. Two milliliters of bacterial suspension was diluted in 15 ml of new KB medium containing 0.3% agar and overlaid on a KB plate. A paper disc (3MM paper; Whatman plc, Brentford, United Kingdom) containing antibiotic (ampicillin at 50 μg/μl or 25 μg/μl or chloramphenicol at 35 μg/μl or 12.5 μg/μl) was placed on the plate. For the H_2O_2 tolerance assay, a nitrocellulose membrane (Millipore Corporation, Bedford, MA) with 20 μl of H_2O_2 solution (7.5% or 15%) was put on the plate. After incubation for 24 h at 27°C, the diameter of the growth inhibition zone was measured.

Quantitative analysis of EPS. Each bacterial strain was grown on an MMMF plate containing 1.5% agar for 48 h at 27°C. The bacterial cells were then harvested and suspended in 200 μl of distilled water. After centrifugation at 8,000 × *g* for 3 min, the supernatant was mixed with 3 volumes of chilled 95% ethanol for 24 h at −20°C, and extracellular polysaccharide (EPS) was precipitated by centrifugation at 8,000 × *g* for 10 min. Quantification of the purified extracellular polysaccharide was carried out by the phenol-sulfate method (15). Total protein in the sample was determined by a Bradford protein assay (Bio-Rad protein assay; Bio-Rad, Hercules, CA). Total extracellular polysaccharide was calculated as a relative value per total cellular protein.

Nucleotide sequence accession numbers. The nucleotide sequence of the *psyI* and *psyR* genes has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB257774.

RESULTS

Detection of AHLs and Northern analysis. To examine whether AHLs are produced by *P. syringae* pv. tabaci 6605, TLC analysis with a C_{18} reverse-phase plate and *C. violaceum* CV026 as the AHL biosensor strain was carried out (Fig. 2A). The production of OHHL and HHL by *P. syringae* pv. tabaci 2024 has been reported, and an OHHL structure was confirmed by mass spectrometry (29). In the WT supernatant from overnight culture in KB, two major signals and one minor signal for violacein were detected. Because the mobilities of the two major signals were consistent with those of the standard molecules, OHHL and HHL, *P. syringae* pv. tabaci 6605 also produces both AHLs (Fig. 2A). The concentration of AHLs in the WT culture medium was estimated at about 0.05 μg/ml for OHHL and at about 0.1 μg/ml for HHL. In contrast, a small amount of AHLs was detected in the Δ*orf3* mutant. Complementation of the strain almost restored production both of HHL and OHHL under these experimental conditions.

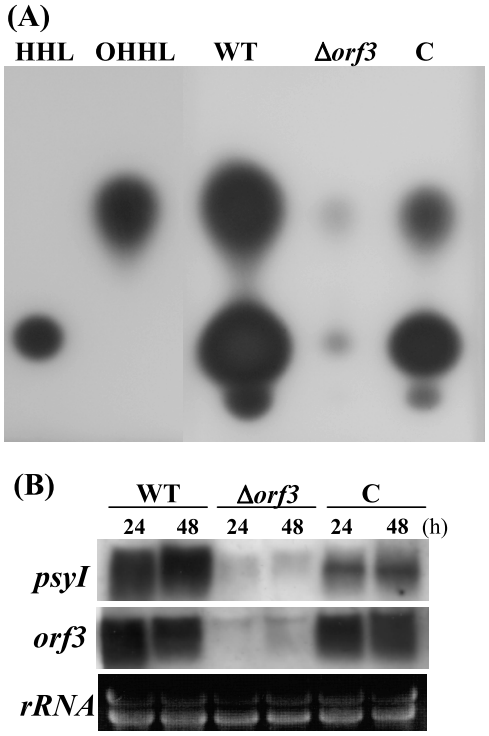

FIG. 2. Detection of AHLs produced by *P. syringae* pv. tabaci and Northern blot analysis. (A) AHL TLC assay. Ten-microliter aliquots of 500-fold-concentrated extracts in ethyl acetate were developed by C_{18} reverse-phase TLC. (B) Expression of *orf3* and *psyI* genes in each strain after 24 and 48 h of incubation in MMMF. HHL and OHHL (0.1 μg each) were used as standards. C, *orf3*-complemented strain.

To ascertain the expression of the *orf3* and *psyI* genes, which encode the AHL synthesis protein in *P. syringae* pv. tabaci 6605, Northern blot analysis was carried out using RNA after 24 and 48 h of incubation in MMMF. As shown in Fig. 2B, the expression of both genes was strong in the WT but was hardly detectable in the Δ*orf3* mutant. In the complemented strain, the expression of *orf3* was recovered to previous levels and that of *psyI* was partially restored.

Biosurfactant detection and colony morphology. The results shown in Fig. 2 suggest that Orf3 is involved in the quorum-sensing system. In some pathogenic bacteria, it has been reported that biosurfactant production is regulated by quorum

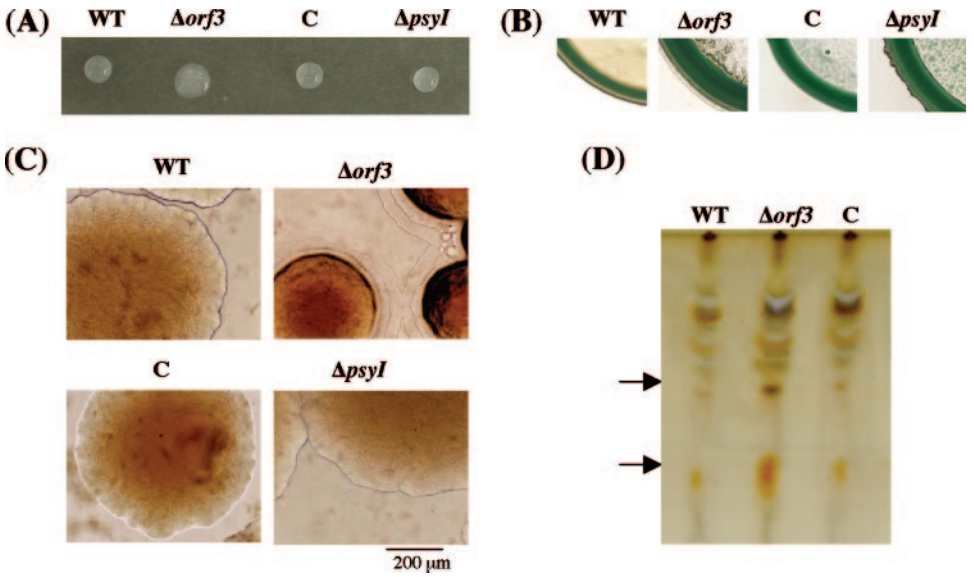

FIG. 3. Detection of biosurfactants, colony morphology, and separation of glycolipids. (A) Drop-collapsing test of bacterial suspension. (B) Methylene blue plate assay. (C) Colony morphology of the WT and each mutant observed by optical microscopy on 1.5% KB agar plate after 2 days of incubation at 27°C. (D) TLC analysis of glycolipids. Arrows indicate orcinol-positive spots with higher intensity in the Δ*orf3* mutant. Bacterial strains are indicated as: C, *orf3*-complemented strain.

sensing (7). Therefore, the characteristics of the WT and Δ*orf3* mutant were examined. Further, to examine whether alternation of the phenotype in the Δ*orf3* mutant is due to a defect in AHL production, a *psyI*-deficient (Δ*psyI*) mutant was generated as a typical mutant defective in AHL production. Loss of ability to produce AHLs by the Δ*psyI* mutant was confirmed by TLC analysis using *C. violaceum* CV026 (data not shown).

The production of biosurfactants was examined by the drop-collapsing test and standard methylene blue plate assay. In the drop-collapsing test, the ability of the Δ*orf3* mutant to produce biosurfactants seemed to be enhanced in comparison with those in WT and complemented strains (Fig. 3A). In the standard methylene blue plate assay, the dark blue ring around the colony of the Δ*orf3* mutant was significantly greater than those produced by WT and complemented strains (Fig. 3B). Although the production of biosurfactants by the Δ*psyI* mutant did not differ from that by the WT in the drop-collapsing test, the Δ*psyI* mutant exhibited enhanced production of biosurfactants in the methylene blue plate assay.

The colony morphology of each strain on KB plates with 1.5% agar was also observed with an optical microscope. The Δ*orf3* mutant showed a somewhat diffused colony whose periphery was surrounded with translucent bilayers, suggesting an overproduction of biosurfactants (Fig. 3C). On the other hand, there was no significant difference in colony morphology among the WT, the complemented strain, and the Δ*psyI* mutant.

The most common class of biosurfactants are glycolipids, consisting of carbohydrates and long-chain aliphatic acids or hydroxyaliphatic acids. Because rhamnolipid, one of the best-studied glycolipids produced in *Pseudomonas aeruginosa* was not detected in the supernatants of overnight cultures of WT *P. syringae* pv. tabaci and the Δ*orf3* mutant in KB medium by the procedure previously reported (31), *P. syringae* pv. tabaci seems not to produce rhamnolipids, at least under these culture conditions. Therefore, glycolipids were extracted from the supernatant with a different solvent, ethyl acetate, and analyzed by TLC. Figure 3D showed detection of glycolipids on the TLC plate with orcinol reagent. The WT, the Δ*orf3* mutant, and its complement strain produced many kinds of glycolipids with different R_f values. Among them, two spots are remarkable in the Δ*orf3* mutant and are candidates for the biosurfactants of this strain.

Swarming motility. Because biosurfactants have been reported to enhance swarming motility (7), the motility of the Δ*orf3* mutant was investigated on 0.5% agar SWM and MMMF plates. As shown in Fig. 4A, the WT, the Δ*psyI* mutant, and the *orf3*-complemented strain showed similar swarming patterns on SWM agar plates. On the other hand, the Δ*orf3* mutant showed a more irregular and branched swarming pattern on SWM plates. Furthermore, only the Δ*orf3* mutant had swarming ability on 0.5% MMMF agar plates (Fig. 4B). The doubling times of the WT and the complemented strain of *P. syringae* pv. tabaci 6605 are 1.03 ± 0.16 h and 1.17 ± 0.14 h, respectively, whereas that of the Δ*orf3* mutant is longer (1.44 ±

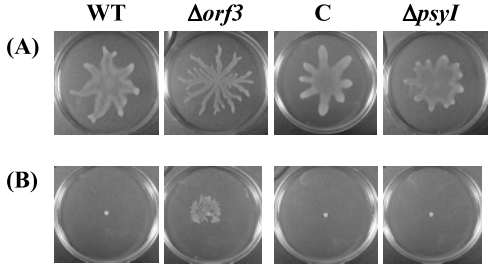

FIG. 4. Swarming motility of *P. syringae* pv. tabaci 6605. Swarming patterns on SWM plates with 0.5% agar after 24 h of incubation at 27°C (A) and on MMMF plates with 0.5% agar after 24 h of incubation at 23°C (B) are shown. C, *orf3*-complemented strain.

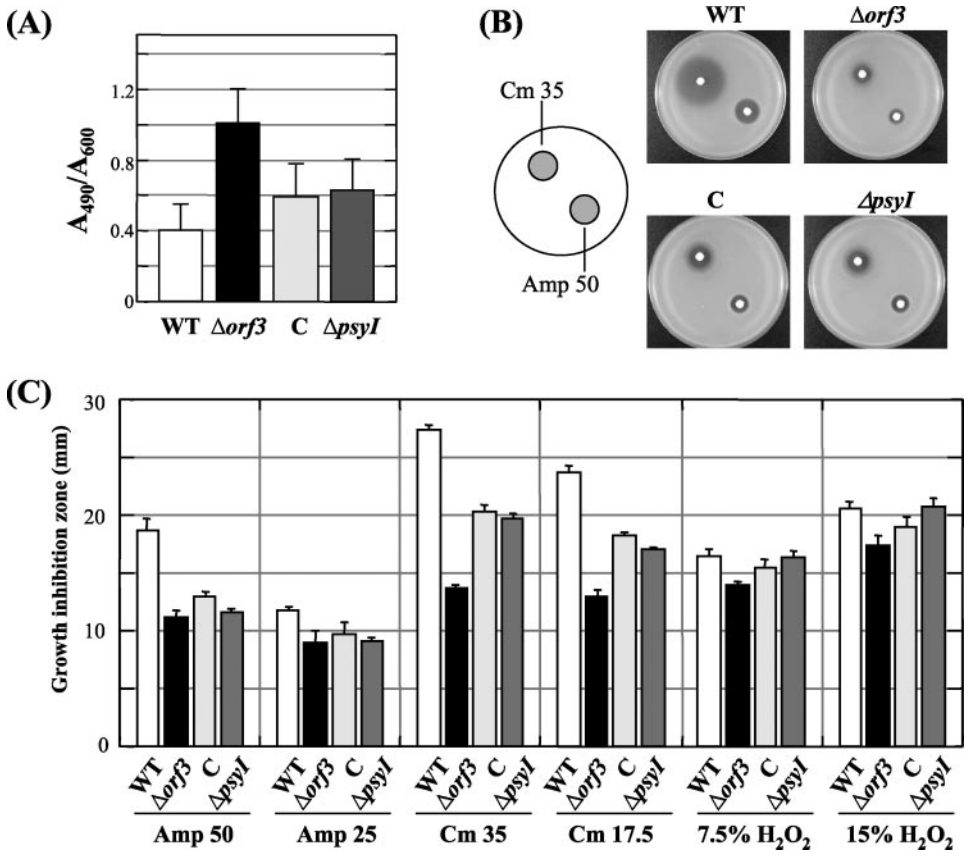

FIG. 5. Extracellular polysaccharide quantification and growth inhibition test. (A) Relative amount of extracellular polysaccharide in bacteria grown on MMMF plate for 48 h at 27°C. (B) Photographs of growth inhibition on plates by 50 μg/μl ampicillin (Amp) and 35 μg/μl chloramphenicol (Cm). (C) Growth inhibition of each bacterial strain by 7.5% and 15% H_2O_2, 50 μg/μl and 25 μg/μl ampicillin, and 35 μg/μl and 17.5 μg/μl chloramphenicol. C, *orf3*-complemented strain. Error bars indicate standard deviations.

0.08 h) and the Δ*psyI* mutant has a slightly longer doubling time (1.27 ± 0.11 h) than the WT, indicating that enhanced swarming ability in the Δ*orf3* mutant was not caused by rapid growth. These results suggest that overproduction of biosurfactants in the Δ*orf3* mutant is one of the causes for the hyperswarming phenotype.

EPS production and tolerance to H_2O_2 and antibiotics. Previous papers have reported that the production of EPS is regulated by quorum sensing and that an EPS-deficient mutant of *P. syringae* is hypersensitive to environmental stresses (7, 17, 25). To compare extracellular polysaccharide production on an MMMF plate with 1.5% agar after 48 h of incubation at 27°C, each mutant was scraped off and the amounts of extracellular polysaccharide and extracellular proteins as an internal control were quantified (Fig. 5A). The results demonstrated that the amount of extracellular polysaccharide in the Δ*orf3* mutant was about twice that in the WT. The extracellular polysaccharide production in the Δ*psyI* mutant was also increased, but weakly. These results suggest that the quorum-sensing system might repress the production of extracellular polysaccharide by *P. syringae* pv. tabaci 6605 under less-nutritional conditions.

The tolerance to H_2O_2 and antibiotics among strains was compared by a growth inhibition test using H_2O_2, ampicillin, or chloramphenicol. The growth inhibition by both ampicillin and chloramphenicol was significantly decreased in the Δ*orf3* mutant in comparison with that in the WT strain, indicating that the Δ*orf3* mutant had increased tolerance to them (Fig. 5B). The degrees of growth inhibition by H_2O_2 and antibiotics in each strain are shown in Fig. 5C. These results indicate that the Δ*orf3* mutant shows higher tolerance to not only H_2O_2 but also to antibiotics and that the Δ*psyI* mutant also has slightly increased tolerance to these substances. Overproduction of EPS and/or biosurfactants may contribute to enhanced tolerance in these mutants.

Production of siderophore. Many bacteria secrete iron-chelating molecules to acquire iron for their own growth. Siderophores are low-molecular-weight iron-chelating compounds, and their production is reportedly controlled by quorum sensing (13, 32). To examine whether siderophore synthesis in *P. syringae* pv. tabaci 6605 is regulated by AHL molecules, the amounts of siderophores in the WT and each mutant were compared using a CAS solution assay.

Figure 6A shows the result of color change due to iron-siderophore complex formation. Because siderophores in the culture supernatant had a high affinity for iron, an orange iron-siderophore complex was formed instead of the blue iron-dye complex. Although all strains produced no or little siderophore at lower cell density, the WT and the complemented strain increased siderophore production in proportion to the increase in cell density (Fig. 6). On the other hand, both the

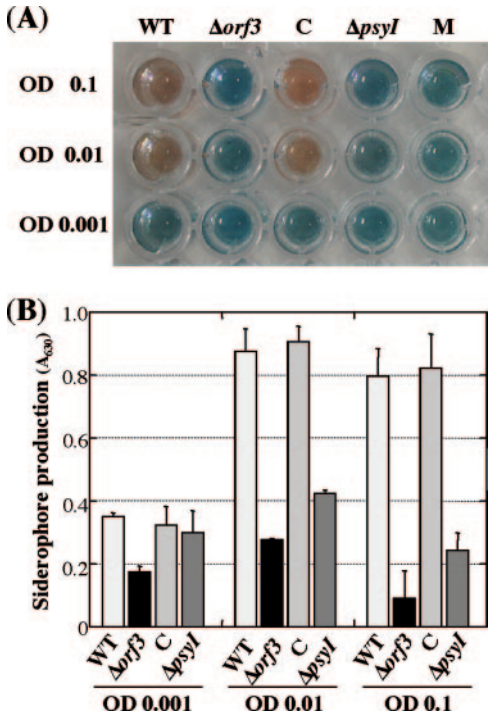

FIG. 6. Siderophore production. (A) Color change of CAS solution containing culture supernatant from each strain. CAS solution with 50 μl of each bacterial supernatant or MMMF as a control (M) were mixed. (B) Quantitative analysis of siderophore production. C, *orf3*-complemented strain. Error bars indicate standard deviations.

Δ*orf3* and Δ*psyI* mutants produced significantly less siderophore, even at higher cell density, suggesting that iron acquisition is regulated by quorum sensing via AHL molecules.

Virulence of mutants on host tobacco leaves and observation of biofilm by scanning electron microscopy. The ability of the WT and the Δ*orf3* and Δ*psyI* mutants to cause disease on host tobacco leaves was examined. The results demonstrate that the Δ*orf3* mutant was less virulent than the WT, as previously reported (33). The Δ*psyI* mutant also showed decreased virulence against tobacco (Fig. 7A).

Bacterial cells on the surfaces of tobacco leaves inoculated with each strain were observed by scanning electron microscopy (Fig. 7B). The WT bacteria were fully embedded in the adhesive EPS matrix, which promoted adhesion to the leaf surface. In contrast, there was little material around the bacterial surface of the Δ*orf3* and Δ*psyI* mutants, and only dried material was observed around them. This result suggests an intimate relationship between AHL production and biofilm formation.

DISCUSSION

In this study, we investigated functions of the *orf3* gene in the glycosylation island of the flagellar gene cluster in *P. syringae* pv. tabaci 6605 by using the *orf3* deletion mutant. Based on homology research, *orf3* was predicted to encode a 3-oxoacyl-ACP synthase III homologue. In the fatty acid biosynthesis pathway of *P. aeruginosa*, acetyl-ACP is derived from acetyl coenzyme A and ACP by FabH (14). In *Lactococcus lactis* subsp. *lactis* IL1403, the homologue of this enzyme was re-

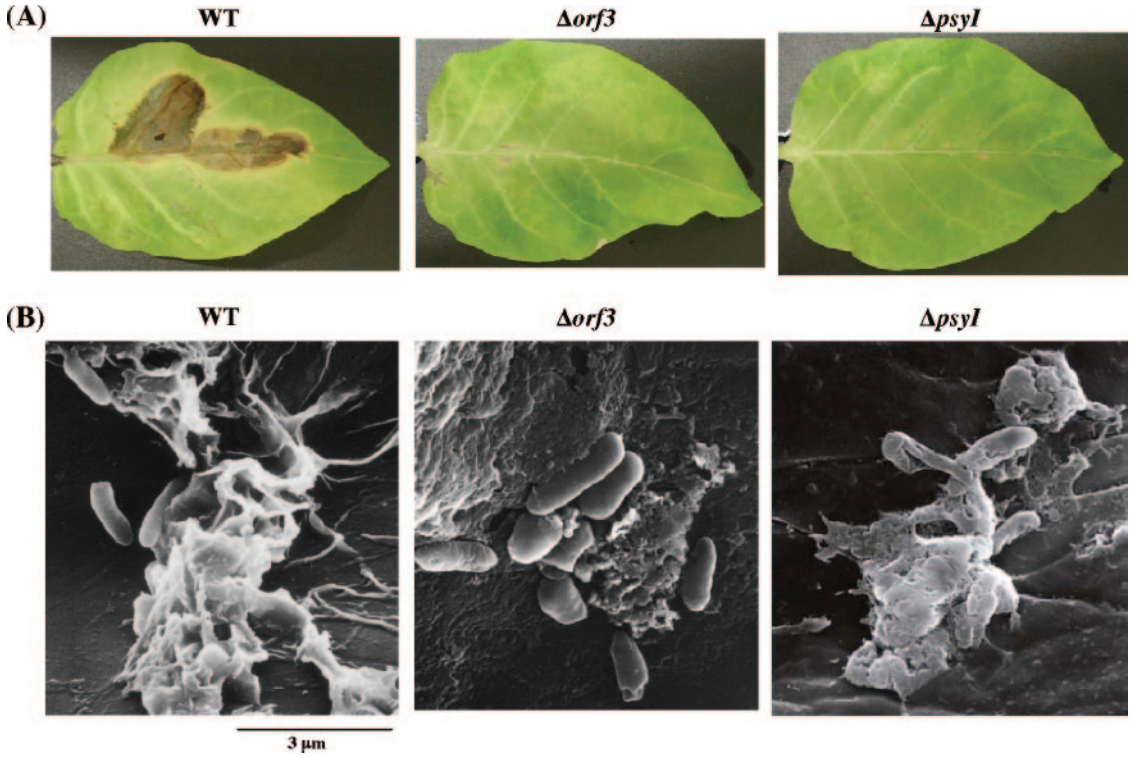

FIG. 7. Bacterial cells on the surfaces of tobacco leaves inoculated with WT *P. syringae* pv. tabaci 6605 and the Δ*orf3* and Δ*psyI* mutants. Leaves were photographed after 8 days of incubation at 23°C (A) and the inoculated tobacco surface was observed by scanning electron microscopy (B).

ported to produce not only acetyl-ACP but also acetoacetyl-ACP (20). Acetyl-ACP is one of the initiators of the fatty acid elongation cycle, and acetoacetyl-ACP is thought to be a precursor of 3-oxo-acyl homoserine lactones. However, because there are several pathways to produce them, FabH is reported to be a dispensable enzyme for biosyntheses of both fatty acids and AHLs in *P. aeruginosa* (14). In contrast, Lai and Cronan (20) reported that FabH is essential for bacterial fatty acid biosynthesis in *E. coli* and *L. lactis* subsp. *lactis* IL-1403 and that FabH-defective mutants failed to grow without exogenous supplementation of long-chain fatty acids.

As shown in Fig. 2, WT *P. syringae* pv. tabaci 6605 synthesized OHHL and HHL as major AHLs, whereas the Δ*orf3* mutant had a significantly reduced ability to produce AHLs. The quorum-sensing system using AHL signals is a recently well-studied bacterial mechanism that unicellular organisms use to communicate with each other and act like multicellular organisms by monitoring their own population density (7). The *orf3* gene is thought to participate in this system by supplementation of AHL precursors. However, the Δ*orf3* mutant produced a small amount of detectable AHLs, suggesting that a minor pathway to produce AHLs may exist in *P. syringae* pv. tabaci 6605, as shown in the previous report for *P. aeruginosa* (14). 3-oxoacyl-ACP synthase I or II may compensate for the function of Orf3. Furthermore, the Δ*orf3* mutant was able to grow in MMMF, suggesting that this enzyme is not indispensable for fatty acid biosynthesis.

Biosurfactants are wetting agents produced by some bacteria to reduce surface tension. The major biosurfactants reported previously are rhamnolipid in *Pseudomonas*, surfactin in *Bacillus*, and serrawettin in *Serratia*, which are glycolipids or lipopeptides. Rhamnolipid production in *P. aeruginosa* is regulated via a quorum-sensing system (4). In the biosynthetic pathway, the 3-ketoacyl-ACP derived from the fatty acid biosynthesis cycle becomes a primer for the subsequent complex steps. Although the structure and de novo biosynthesis pathway of biosurfactants of *P. syringae* pv. tabaci 6605 have not been elucidated, we detected biosurfactant production by this bacterium (Fig. 3). In particular, the production of biosurfactants in the Δ*orf3* mutant was significantly higher than those in the WT and the Δ*psyI* mutant. Although some candidates for biosurfactants were detected (Fig. 3D), their structures have not yet been determined. Structural analysis of these compounds by mass spectrometry and nuclear magnetic resonance will be needed in near future.

Why is the production of biosurfactants facilitated in the Δ*orf3* mutant? The answer to this question is not clear at present. However, if Orf3 catalyzes the rate-limiting step of fatty acid biosynthesis, short-chain ACPs such as malonyl-ACP, substrates for Orf3, will be accumulated in the Δ*orf3* mutant. In this case, the enzymes for biosurfactant production may be able to use these short-chain ACPs without competition.

Previously, it was reported that swarming motility is enhanced by the addition of biosurfactants and that mutants of *Serratia liquefaciens* defective in biosurfactant production have no swarming ability (19, 21). Indeed, swarming motility in the Δ*orf3* mutant was enhanced in both SWM and MMMF plates with 0.5% agar (Fig. 4). The Δ*psyI* mutant showed the same level of swarming ability as the WT, although AHL-deficient mutants of *P. syringae* pv. syringae B728a exhibited high motility (25). Because this mutant is defective in both the *ahlI* and *ahlR* genes, which encode an AHL synthetic enzyme and a transactivator of AHL-responsive genes, the phenotype might be different from that caused by our single mutation of *psyI* in *P. syringae* pv. tabaci 6605. Further investigation of the regulation of swarming ability is required.

It was reported that a *P. syringae* mutant defective in EPS production was hypersensitive to H_2O_2 (17, 25). In *P. syringae* pv. tabaci 6605, tolerance to H_2O_2 and antibiotics was enhanced in the Δ*orf3* mutant, probably owing to the overproduction of biosurfactants and/or EPS (Fig. 3 and 5). Our results suggest that EPS and/or biosurfactants are important for swarming motility and tolerance to environmental stresses. Thus, the quorum-sensing system in *P. syringae* pv. tabaci 6605 is thought to negatively regulate their production.

Iron uptake using siderophores has been reported to be regulated by the quorum-sensing system in *P. aeruginosa* (32). Because environmental iron is almost insoluble at biological pH, many bacteria have developed systems to acquire iron by using siderophores, which show high affinity for iron(III) (28). As shown in Fig. 6, both the Δ*orf3* and the Δ*psyI* mutants showed a drastically reduced ability to produce siderophores even at high bacterial density. This result suggests that siderophore production is positively regulated via the quorum-sensing system in *P. syringae* pv. tabaci 6605.

Many species of pseudomonads produce a fluorescent yellow-green siderophore called pyoverdine. Fluorescent pseudomonads are also able to produce other, minor siderophores such as pyochelin, pseudomonine, quinolobactin, and corrugatin (6). Recently, it was reported that pyoverdine is generally detected by many pathovars of *P. syringae*, although the spectral characteristics are different from those of typical pyoverdine in animal pathogens (5). WT *P. syringae* pv. tabaci 6605 has greater ability to produce fluorescence under UV light than the Δ*orf3* and Δ*psyI* mutants (data not shown), suggesting that pyoverdine may be the major siderophore in this bacterium. Indeed, there are highly homologous genes for pyoverdine side chain peptide synthase in *P. syringae* pv. tabaci 6605 (data not shown).

Recently, gene expression profiles of *S. enterica* serovar Typhimurium during swarming were compared with those in liquid medium by microarray analysis (37). The results demonstrated that genes for iron metabolism were strongly induced in bacteria grown on swarming agar plates with less-nutritional conditions. It was reported that excess iron prevents swarming motility, and less-nutritional conditions may induce swarming and biosurfactant production in *P. aeruginosa* (7). From these reports, a reduced ability to acquire iron may relate to the hyperswarming motility in the Δ*orf3* mutant.

Iron acquisition and biosurfactant production were reported to influence biofilm formation (2, 3). Normal biofilm is composed of bacterial cells and EPS, with large amounts of water in its structure (10). However, when a WT strain of *P. aeruginosa* was incubated with lactoferrin, an iron chelator, a thick, mushroom-like structured biofilm was not observed by confocal scanning laser microscopy (2). The siderophore-defective mutant also formed only a thin uniform layer (2). Furthermore, overproduction of biosurfactants inhibited biofilm development in *P. aeruginosa* (8), probably because bacterial

detachment from the biofilm occurred earlier and more extensively (3). As shown in Fig. 7, both the Δ*orf3* and Δ*psyI* mutants had reduced virulence toward the host tobacco leaves, and the biofilm formation by each strain seemed not to develop normally. To elucidate iron functions as a signal for swarming and biofilm development, we now plan to construct a mutant of *P. syringae* pv. tabaci 6605 that is defective in iron acquisition by disruption of related genes of pyoverdine synthesis. In addition, further characterization of the genes responsible for biosurfactant synthesis is required.

We investigated the *orf3*-mediated regulation of virulence factors in *P. syringae* pv. tabaci 6605. Why is *orf3*, a gene concerned with quorum sensing, located in the glycosylation island of the flagellar gene cluster? There are two stages in the process of bacterial attachment: the primary docking stage and the secondary locking stage (11). In the primary docking stage, bacteria must approach the surface against electrostatic and hydrophobic forces via swimming motility and chemotaxis. During this stage, at lower cell density, flagellum-mediated motility is thought to play an important role; however, EPS, if any, may obstruct active flagellar motility. In the subsequent secondary locking stage, loosely bound bacteria attach firmly to the surface by producing EPS for biofilm maturation. In this stage, EPS becomes an essential factor, but flagellum-mediated motility might not be necessary. After sufficient maturation of the biofilm, bacteria begin to escape from the old biofilm and colonize other surfaces. The flagellum-mediated swarming motility might be induced at this detachment process owing to the limited availability of nutrients, including iron, in the mature biofilm. Recent papers have suggested that each process of biofilm formation might be regulated by expression of quorum-sensing genes (1, 9). These dynamic alternations in gene expression for biofilm formation might be regulated by the *orf3* gene in relation to flagellum expression.

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