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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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Title: **Homologue of 3-oxoacyl-(acyl carrier protein) synthase III gene located in glycosylation island of *Pseudomonas syringae* pv. *tabaci* regulates virulence factors via *N*-acyl homoserine lactone and fatty acid synthesis**

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iv) Running title: Orf3 mediates regulation of quorum sensing

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Abstract

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 post-translational modification of flagellin. Although the function of Orf3 remained unclear, an *orf3*-deletion mutant (Δ*orf3*) 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 significantly reduced ability to form acyl-homoserine lactones (AHLs), quorum sensing molecules, suggesting that Orf3 is required for AHL synthesis. In comparison with the wild-type (WT) 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 mutant (Δ*psyI*) 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 in 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.

Introduction

Pseudomonas syringae pv. *tabaci* 6605, an isolate of a phytopathogenic bacteria, causes wildfire disease on host tobacco plants, and induces 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 of *P. syringae* pv. *glycinea* does induce HR on tobacco plant, suggesting that post-translational 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 *orfs*, namely *orf1, orf2* and *orf3,* in the glycosylation island of 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 deletion mutant of *orf1* and *orf2* genes (Δ*orf1* and Δ*orf2*) 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 post-translational modification (33).

A homology search revealed that *orf3* is highly homologous to the putative 3-oxoacyl-(acyl carrier protein (ACP)) synthase III (ß-ketoacyl-ACP synthase III, KAS III) of *Escherichia coli, Salmonella enterica* serover typhimurium, and *Pseudomonas putida* strain KT2440 (24, 36). It is reported that 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 is 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 structure 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 (SAM) and acyl chains from the acyl-ACP pool in 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 mutant Δ*psyI*, and compared its characteristics with 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 (LB) medium. *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 x 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 mutant (Δ*psyI*), the genetic region of *psyI* and *psyR* was first isolated by a TA cloning system (pGEMT-Easy, Promega, Tokyo, Japan). PCR primers (PsyI5': 5'-ATGTCGAGCGGGTTTGAGTTTCAG-3'; 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 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 original volume of ethyl acetate, and 10 µl of the solution was subjected to C18 reversed-phase thin layer chromatography (TLC, 20 cm x 20 cm, RP-18F254S, Merck, Darmstadt, Germany) with a solvent system of methanol and water (60:40, v/v). After development, the dried TLC plate was overlaid with 50 ml of semi-solid LB agar medium containing 6 ml of an overnight culture of *C. violaceum* CV026. After 24 h 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 the gift of Prof. T. Ikeda (Utsunomiya University). All samples were stored at -20°C in ethyl acetate.

Northern blot analysis

Pseudomonas strains were incubated in LB medium 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 medium) (30) and incubated to an 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 DIG Synthesis Kit (Roche). The conditions of hybridization and detection followed the methods of Shimizu et al. (30).

Scanning electron and optical microscopy

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

Swarming 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 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, USA) (18) or minimal medium containing 10mM mannitol and fructose (MMMF plate with 0.5% agar) (33). The swarming motility was observed after 24 h incubation at 27°C for SWM and at 23°C for MMMF agar plates.

Biosurfactant assay

Overnight culture 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 supernatant were spotted on the film (Parafilm, Alcan Packaging, Neenah, WI, USA) to detect the drop-collapsing activity (21).

For biosurfactant detection, overnight culture in LB medium 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 (HAB), as described by Kohler et al. (19). The plate was incubated for 48 h at 27°C until the appearance of a blue halo.

Glycolipids 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 filtrated through a 0.45 µm pore-size filter and glycolipids were extracted by equal volume of ethyl acetate. The organic phase was evaporated to dryness and resuspended in 400 µl of ethyl acetate. Fifteen microliters of sample were subjected to TLC on pre-coated silica gel glass plate (SILICA GEL 60, Whatman Inc. Clifton, NJ, USA) with a solvent system of chloroform : methanol : water (95 : 20 : 2 v/v/v). After development glycolipids were visualized by spraying with 0.2% orcinol reagent dissolved in 11.2% H_2SO_4 at a final concentration and heating at 110°C for 15 min (31).

Chromoazurol S assay

The siderophore production of 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 incubation, each culture was filtrated 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 HAB, 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 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* bacterial strains were grown for 24 h in KB. Two ml of bacterial suspension was diluted in 15 ml of new KB medium containing 0.3% agar and overlaid on a KB plate. Paper disc (3MM paper, Whatman plc, Brentford, UK) containing antibiotic (ampicillin at 50 µg/µl or 25 µg/µl, chloramphenicol at 35 µg/µl or 12.5 µg/µl) was placed on the plate. For H_2O_2 tolerance assay, the nitrocellulose membrane (Millipore Corporation, Bedford, MA, USA) 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 a growth inhibition zone was measured.

Quantitative analysis of extracellular polysaccharide

Each bacterial strain was grown on an MMMF plate containing 1.5% agar for 48 h at 27°C. Then bacterial cells were harvested and suspended in 200 µl of distilled water. After centrifugation at 8000 x g for 3 min, the supernatant was mixed with three volumes of chilled 95% ethanol for 24 h at -20°C, and extracellular polysaccharide was precipitated by centrifugation at 8000 x 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* gene has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB257774.

Results

Detection of AHLs and Northern analysis

To examine whether AHLs are produced by *P. syringae* pv. *tabaci* 6605, TLC analysis by a C18 reverse-phase plate using *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 over night culture in KB, two major and one minor signals 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 about 0.1 μg/ml for HHL. In contrast, a slight amount of AHLs was detected in the Δ*orf3* mutant. Complementation of the strain almost restored production both of HHL and OHHL in this experimental condition.

To ascertain the expression of *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 incubation in MMMF medium. 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 is reported that biosurfactant production is regulated by quorum 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 of AHL production, a *psyI*-deficient mutant (Δ*psyI*) was generated as a typical defective mutant of 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 of 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 is no significant difference in colony morphology among WT, complemented strain, and the Δ*psyI* mutant.

The most common class of biosurfactants is glycolipids, consisting of carbohydrates and long-chain aliphatic acids or hydroxyaliphatic acids. Because rhamnolipid, one of the best-studied glycolipids produced in *P. aeruginosa* was not detected in the supernatant of overnight culture of *P. syringae* pv. *tabaci* WT and Δ*orf3* mutant in KB medium with the procedure previously reported (31), *P. syringae* pv. *tabaci* seems not to produce rhamnolipids at

least in this culture condition. Therefore, glycolipids were extracted from the supernatant with different solvent, ethyl acetate, and analyzed with TLC. Fig. 3D showed detection of glycolipids on the TLC plate with orcinol reagent. WT, Δ*orf3* mutant and its complement strain produced many kinds of glycolipids with different R_f value. Among them two spots indicated with arrows are remarkable in Δ*orf3* mutant, and are the candidates for the biosurfactants of this strain.

Swarming motility

Because biosurfactants are 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, Δ*psyI* mutant, and *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 time of WT and complemented strain of *P. syringae* pv. *tabaci* 6605 is 1.03±0.16 h and 1.17±0.14 h, respectively. Whereas that of the Δ*orf3* mutant is longer (1.44±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 hyper-swarming phenotype.

EPS production and tolerance to H_2O_2 and antibiotics

Previous papers reported that the production of EPS is regulated by quorum sensing and EPS-deficient mutant of *P. syringae* is hyper-sensitive to environmental stresses (7, 17, 25). To compare extracellular polysaccharide production on an MMMF plate with 1.5% agar after 48 h incubation at 27°C, each mutant was scraped off and the amount of extracellular polysaccharide and extracellular proteins as an internal control was quantified (Fig. 5A). The result 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 chloramphenocol. The growth inhibition by both of 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 degree of growth inhibition by H_2O_2 and antibiotics in each strain is 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 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. Siderophore is a low molecular weight iron-chelating compound, and its 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 amount of siderophore in the WT and each mutant was compared using a CAS solution assay.

Fig. 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-colored iron-siderophore complex was formed instead of the blue colored iron-dye complex. Although all strains produced no or little siderophore at lower cell density, the WT and complemented strain increased siderophore production in proportion to the increase in cell density (Fig. 6). On the other hand, both the 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 Δ*orf3* and Δ*psyI* mutants to cause disease on host tobacco leaves was examined. The result demonstrates 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 surface 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 the 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 flagella gene cluster in *P. syringae* pv. *tabaci* 6605 using the *orf3*-deletion mutant. Based on the 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-CoA and ACP by FabH (14). In the *Lactococcus lactis* subspecies *lactis* IL1403, the homologue of this enzyme was reported to produce not only acetyl-ACP but also acetoacetyl-ACP (20). The 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 both of biosyntheses of fatty acid 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 the *L. lactis* subspecies *lactis* IL1403 and that FabH-defective mutants failed to grow without exogenous supplementation of long-chain fatty acids.

As shown in Fig. 2, the WT of *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 multi-cellular 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). Probably, 3-oxoacyl-ACP synthase I or II may compensate for the function of Orf3. Furthermore, the Δ*orf3* mutant was able to grow in the MMMF medium, suggesting that this enzyme is not indispensable for fatty acids 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 Δ*psyI* mutant. Although some candidates for biosurfactants were detected (Fig. 3D), their structures were not determined yet. Structural analysis of these compounds with 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 from *P. syringae* pv. *syringae* B728a exhibited high motility (25). Because this mutant is defective in both *ahlI* and *ahlR* genes, which encode AHL synthetic enzyme and transactivator of AHL-responsive genes, the phenotype might be different from 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 (Figs. 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 these production.

Iron up-take 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 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 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 pseudomonad produce 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 pathogen (5). *P. syringae* pv. *tabaci* 6605 WT has higher ability to produce fluorescence under UV light than the Δ*orf3* and the Δ*psyI* mutants (data not shown), suggesting pyoverdine may be the major siderophore in this bacterium. Indeed, there is highly homologous genes for pyoverdine side chain peptide synthase in *P. syringae* pv. *tabaci* 6605 (data not shown).

Recently, gene expression profiles of *S. typhimurium* during swarming were compared with those in liquid media by microarray analysis (37). The result 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, reduced ability to acquire iron may relate to the hyper-swarming 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 the WT 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* (9), probably because bacterial detachment from the biofilm occurred earlier and more extensively (3). As shown in Fig. 7, both the Δ*orf3* and the Δ*psyI* mutants had reduced virulence toward the host tobacco leaves, and the biofilm formation of 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 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 flagella 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, flagella-mediated motility is thought to play an important role; however, EPS, if any, may obstruct active flagella motility. In the subsequent secondary locking stage, loosely bound bacteria are attached firmly to the surface by producing EPS for biofilm maturation. In this stage, EPS becomes an essential factor, but flagella-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 flagella-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 suggested that each process of biofilm formation might be regulated by expression of quorum-sensing genes (1, 8). These dynamic alternations in gene expression for biofilm formation might be regulated by the *orf3* gene in relation to flagella expression.

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Figure legends

FIG. 1. Schematic organization of 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 flgaellin-glycosylation, *orf3*: putative gene encoding 3-oxoacyl-(acyl carrier protein) synthase III, *fliC*: gene encoding flagellin. Arrows indicate putative transcripts and directions of transcription.

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 C18 reversed-phase TLC. (B) Expression of *orf3* and *psyI* genes in each strain after 24 and 48 h incubation in MMMF medium. HHL and OHHL (0.1 µg each) are used as standards. Bacterial strains are indicated as: wild-type (WT), Δ*orf3*, and *orf3*-complemented strain (C).

FIG. 3. Detection of biosurfactants, colony morphology, and separation of glycolipids (A) The drop-collapsing test of bacterial suspension. (B) Methylene blue plate assay. (C) Colony morphology of WT and each mutant observed by optical microscopy on 1.5% KB agar plate after 2 days 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: wild-type (WT), Δ*orf3*, Δ*psyI*, and *orf3*-complemented strain (C).

FIG. 4. Swarming motility of *P. syringae* pv. *tabaci* 6605. Swarming patterns on SWM plates with 0.5% agar after 24 h incubation at 27°C (A) and on MMMF plates with 0.5% agar after 24 h incubation at 23°C (B). Bacterial strains are indicated as: wild-type (WT), Δ*orf3*, Δ*psyI*, and *orf3*-complemented strain (C).

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 plate 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. Bacterial strains are indicated as: wild-type (WT), Δ*orf3*, Δ*psyI*, and *orf3*-complemented strain (C).

FIG. 6. Siderophore production. (A) The color change of CAS solution containing culture supernatant from each strain. CAS solution with 50 µl of each bacterial supernatant or MMMF medium as a control (M) were mixed. (B) A quantitative analysis of siderophore production. Bacterial strains are indicated as: wild-type (WT), Δ*orf3*, Δ*psyI*, and *orf3*-complemented strain (C).

FIG. 7. Bacterial cells on the surface of tobacco leaves inoculated with *P. syringae* pv. *tabaci* 6605 WT, Δ*orf3*, and Δ*psyI* mutants. Leaves were photographed after 8 days incubation at 23°C (A), and the inoculated tobacco surface was observed by scanning electron micrograph (B). The bars represent 3 µm.

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⁻ø80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK⁻ mK⁺) supE44 thi-1 gyrA relA1*	Takara, Kyoto, Japan
S17-1	*thi pro hsdR⁻hsdM⁺recA [chr::RP4-2-Tc::Mu-Km::Tn7]*	26
Chromobacterium violaceum CV026	Double mini-Tn*5* mutant from *C. violaceum* ATCC31532, AHL biosenser	22
Pseudomonas syringae pv. *tabaci*		
Isolate 6605	Wild type, Nal^r	30
6605-d3	Isolate 6605 Δ*orf3*	33
6605-dpsyI	Isolate 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 *Eco*RI 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

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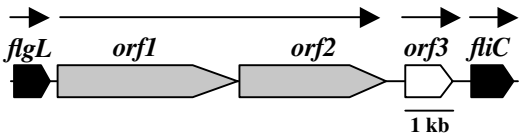
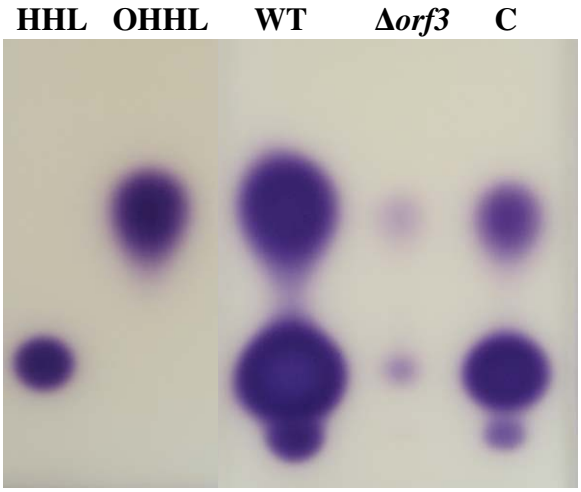
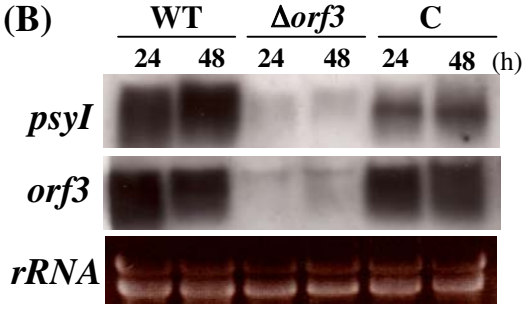
Fig. 1

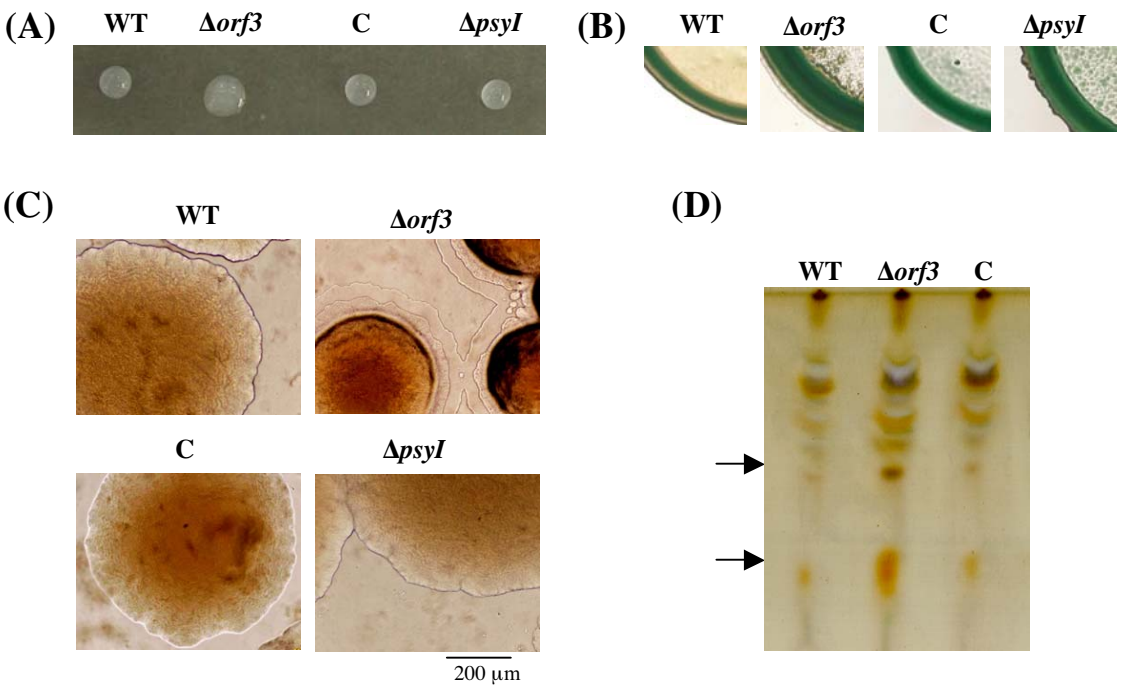


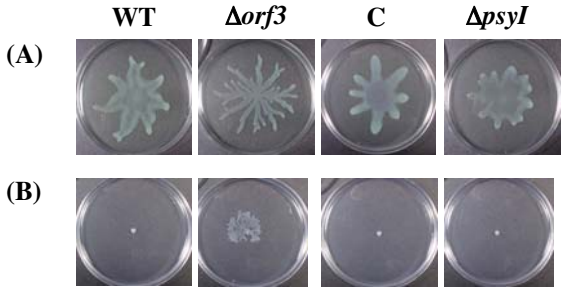
(A)

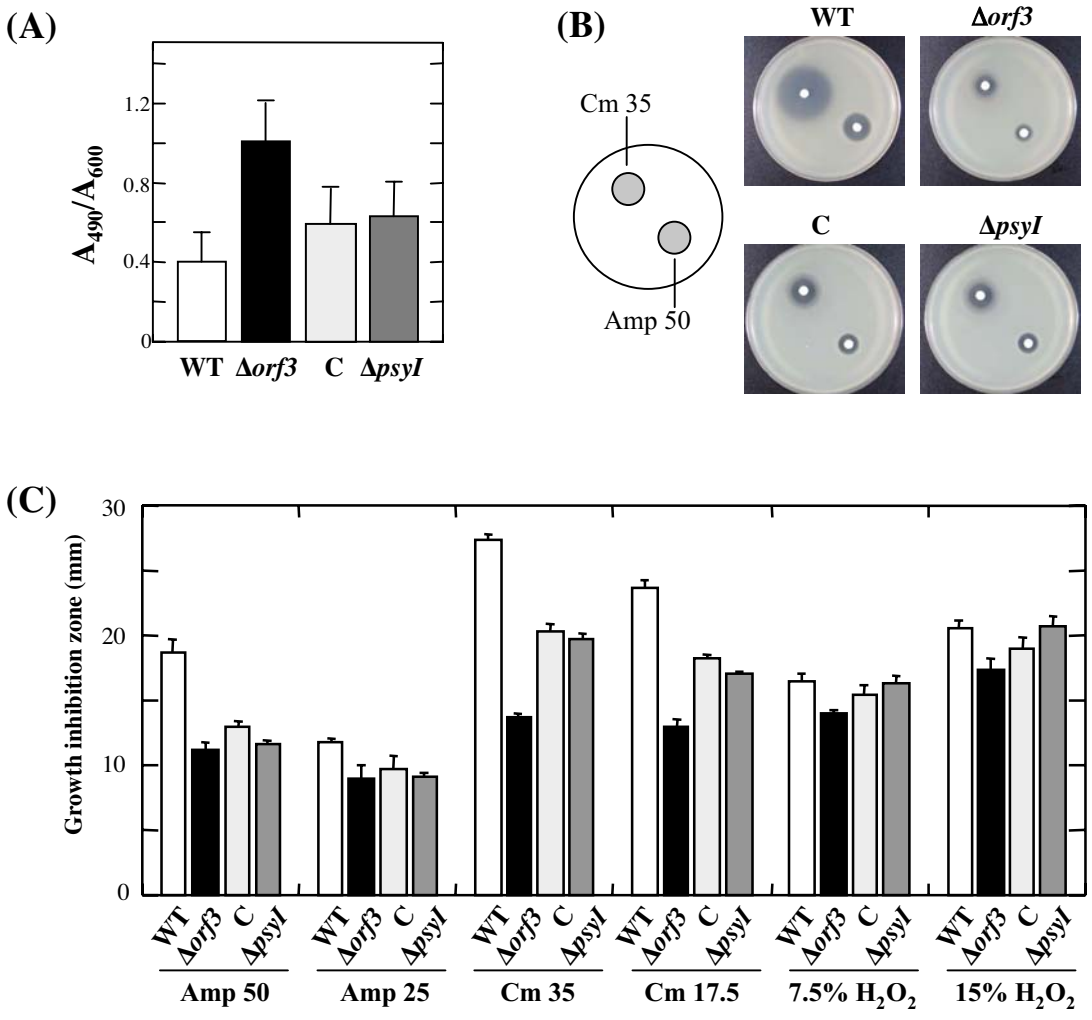


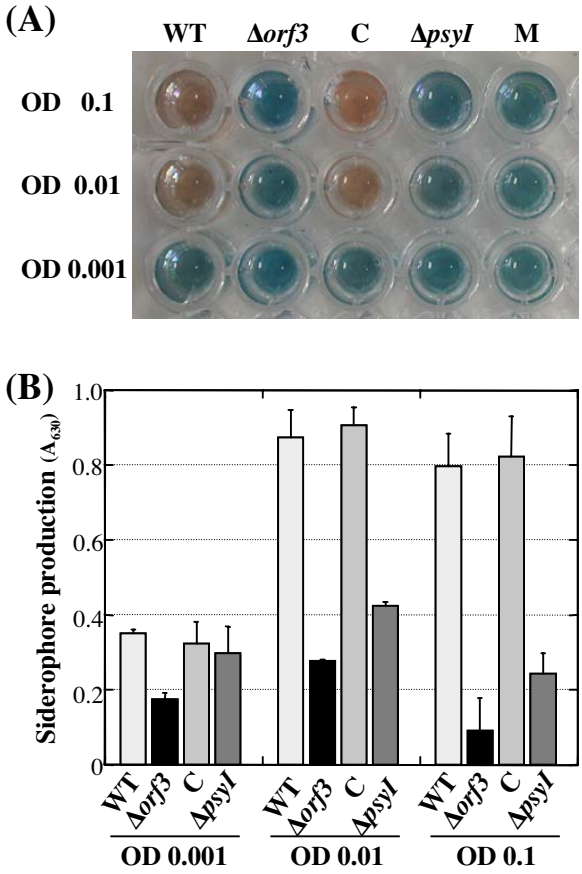
(B)

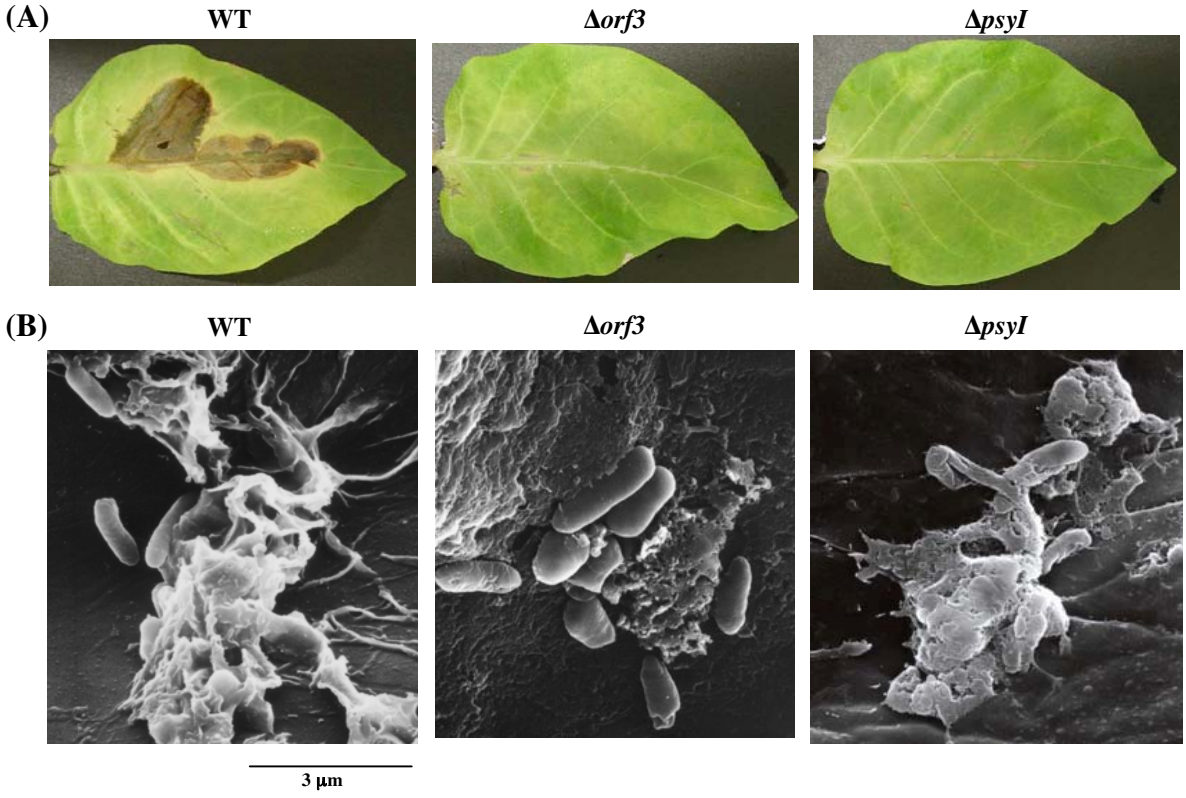



(A)
WT
Δorf3
C
ΔpsyI
(B)
WT
Δorf3
C
ΔpsyI
(C)
WT
Δorf3
C
ΔpsyI
200 μm
(D)
WT
Δorf3
C


WT
Δorf3
C
ΔpsyI
(A)
(B)


(A)
A_{490}/A_{600}
1.2
0.8
0.4
0
WT
Δorf3
C
ΔpsyI
(B)
Cm 35
Amp 50
WT
Δorf3
C
ΔpsyI
(C)
Growth inhibition zone (mm)
30
20
10
0
WT
Δorf3
C
ΔpsyI
Amp 50
Amp 25
Cm 35
Cm 17.5
7.5% H_2O_2
15% H_2O_2


(A)
WT
Δorf3
C
ΔpsyI
M
OD 0.1
OD 0.01
OD 0.001
(B)
Siderophore production (A630)
1.0
0.8
0.6
0.4
0.2
0
WT
Δorf3
C
ΔpsyI
OD 0.001
OD 0.01
OD 0.1


(A)
WT
Δorf3
ΔpsyI
(B)
WT
Δorf3
ΔpsyI
3 μm