

Complete Genome Sequence of *Pseudomonas chlororaphis* subsp. *aurantiaca* Reveals a Triplicate Quorum-Sensing Mechanism for Regulation of Phenazine Production

TOMOHIRO MOROHOSHI^{1*}, TAKAHITO YAMAGUCHI¹, XIAONAN XIE², WEN-ZHAO WANG³, KASUMI TAKEUCHI⁴, and NOBUTAKA SOMEYA⁵

¹Department of Material and Environmental Chemistry, Graduate School of Engineering, Utsunomiya University, 7–1–2 Yoto, Utsunomiya, Tochigi 321–8585, Japan; ²Center for Bioscience Research and Education, Utsunomiya University, 350 Mine-machi, Utsunomiya, Tochigi 321–8505, Japan; ³Key Laboratory for Eco-Efficient Polysilicate Materials, School of Environment and Energy, Peking University Shenzhen Graduate School, Guangdong 518055, China; ⁴Division of Plant and Microbial Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 2–1–2 Kannondai, Tsukuba, Ibaraki 305–8602, Japan; and ⁵Institute of Vegetable and Floriculture Sciences, National Agriculture and Food Research Organization, 3–1–1 Kannondai, Tsukuba, Ibaraki 305–8519, Japan

(Received October 28, 2016—Accepted December 17, 2016—Published online February 25, 2017)

Pseudomonas chlororaphis subsp. *aurantiaca* StFRB508 regulates phenazine production through *N*-acyl-L-homoserine lactone (AHL)-mediated quorum sensing. Two sets of AHL-synthase and AHL-receptor genes, *phzI/phzR* and *aurI/aurR*, have been identified from the incomplete draft genome of StFRB508. In the present study, the complete genome of StFRB508, comprising a single chromosome of 6,997,933 bp, was sequenced. The complete genome sequence revealed the presence of a third quorum-sensing gene set, designated as *csaI/csaR*. An LC-MS/MS analysis revealed that StFRB508 produced six types of AHLs, with the most important AHL being *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-OH-C6-HSL). PhzI mainly catalyzed the biosynthesis of 3-OH-C6-HSL, while AurI and CsaI catalyzed that of *N*-hexanoyl-L-homoserine lactone and *N*-(3-oxohexanoyl)-L-homoserine lactone, respectively. A mutation in *phzI* decreased phenazine production, whereas that in *aurI* or *csaI* did not. A *phzI aurI csaI* triple mutant (508ΔPACI) did not produce phenazine. Phenazine production by 508ΔPACI was stimulated by exogenous AHLs and 3-OH-C6-HSL exerted the strongest effects on phenazine production at the lowest concentration tested (0.1 μM). The plant protection efficacy of 508ΔPACI against an oomycete pathogen was lower than that of wild-type StFRB508. These results demonstrate that the triplicate quorum-sensing system plays an important role in phenazine production by and the biocontrol activity of StFRB508.

Key words: *Pseudomonas chlororaphis*, quorum sensing, acylhomoserine lactone, phenazine, biocontrol

Phenazine and its derivatives are orange-pigmented heterocyclic products of bacterial secondary metabolism, and are known for their broad-spectrum antifungal activity (12, 18). A wide variety of phenazine derivatives, such as phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), are produced by various Gram-negative and Gram-positive genera, including *Brevibacterium*, *Burkholderia*, *Pseudomonas*, and *Streptomyces* (18). The core phenazine biosynthetic gene cluster contains seven genes (*phzABCDEFG*) and is present in several *Pseudomonas* species such as *Pseudomonas chlororaphis*, *P. aeruginosa*, and *P. fluorescens* (12). Phenazine-producing *Pseudomonas* species have been studied as biocontrol agents against many plant diseases (27). *P. chlororaphis* PCL1391 produces PCN and controls tomato foot and root rot caused by *Fusarium oxysporum* (3). PCN and PCA produced by *P. aeruginosa* PNA1 are involved in the biocontrol of *Pythium myriotylum* on cocoyam (24).

Quorum sensing is a bacterial cell-cell communication process and modulates cell density-dependent gene expression (16). *N*-Acyl-L-homoserine lactone (AHL) has been identified as a signaling compound involved in quorum-sensing

in many Gram-negative bacteria (16). The LuxI/LuxR system is used by many Gram-negative bacteria to control the expression of genes regulated by quorum sensing (16). Members of the LuxI protein family catalyze AHL biosynthesis. When AHL concentrations reach a threshold due to increases in bacterial cell density, members of the LuxR protein family, comprising AHL receptors, bind to AHL, thereby regulating the expression of many genes. Previous studies reported that the production of some antimicrobial compounds, such as carbapenem, prodigiosin, and pyrrolnitrin, is regulated by AHL-mediated quorum sensing (21, 25). The expression of the phenazine biosynthesis gene cluster in fluorescent pseudomonads is activated in the stationary growth phase in a manner that depends on the production of AHLs (17). The quorum sensing-mediated regulation of the phenazine biosynthesis gene cluster in some *P. chlororaphis* strains has been investigated in detail (3, 7, 27). AHLs are mainly synthesized by PhzI in phenazine-producing *P. chlororaphis* (27). The AHL receptor protein, PhzR, binds to AHLs and activates the expression of the phenazine biosynthetic gene cluster. Another quorum-sensing system, *csaI/csaR*, was detected in *P. chlororaphis* subsp. *aureofaciens* 30-84, but was not involved in the regulation of the phenazine biosynthetic gene cluster (27). We previously isolated *P. chlororaphis* subsp.

* Corresponding author. E-mail: morohosi@cc.utsunomiya-u.ac.jp;
Tel: +81–28–689–6176; Fax: +81–28–689–6176.

aurantiaca StFRB508 from potato roots and identified two functional quorum-sensing systems, *phzI/phzR* and *aurI/aurR*, in the incomplete draft genome sequence of StFRB508 (13). The findings of a thin-layer chromatography (TLC) analysis revealed that PhzI catalyzes the biosynthesis of *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-OH-C6-HSL), while AurI catalyzes that of *N*-butanoyl-L-homoserine lactone (C4-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL). Phenazine production in AHL-negative mutants was stimulated by exogenous AHLs and the most active AHL was 3-OH-C6-HSL. In an attempt to elucidate the quorum-sensing system involved in the regulation of phenazine production in the present study, the complete genome of StFRB508 was sequenced and a third quorum-sensing system was identified. In addition, the concentrations and structures of AHLs produced by StFRB508 were accurately measured and elucidated using LC-MS/MS. We also investigated the biocontrol activity of StFRB508 and its quorum-sensing-deficient mutant.

Materials and Methods

Bacterial strains, compounds, and growth conditions

The bacterial strains used in this study are listed in Table S1. All bacterial strains were grown in Luria-Bertani (LB) medium (10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, and 5 g L⁻¹ NaCl). Solid bacterial media were prepared by the addition of agar at a final concentration of 1.5%. Antibiotics were added as required at final concentrations of 100 µg mL⁻¹ carbenicillin and 50 µg mL⁻¹ kanamycin. The AHLs used in this study, C6-HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), were synthesized using a previously described method (2). 3-OH-C6-HSL, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-OH-C8-HSL), *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-OH-C10-HSL), and *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (3-OH-C12-HSL) were prepared from 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, and 3-oxo-C12-HSL, respectively, via the reduction of the ketone functional group with NaBH₄ by a previously described method (19).

Genome sequencing of StFRB508

The single- and paired-end whole-genome shotgun sequencing of StFRB508 was performed using a Roche Genome Sequencer FLX Titanium pyrosequencer (Eurofins Genomics, Tokyo, Japan) as described in a previous study (13). Gap closure was attempted using gap-spanning clones and PCR products. The prediction of putative coding sequences and gene annotation were performed using the Microbial Genome Annotation Pipeline (<http://www.migap.org/>). Briefly, protein-coding sequences (CDSs) were predicted by the combined use of MetaGeneAnnotator (14), RNAmmer (8), tRNAScan (11), and BLAST (1).

Cloning and disruption of quorum-sensing genes of StFRB508

The *phzIR*, *aurIR*, and *csaIR* coding regions on the StFRB508 genome were amplified with Blend Taq plus DNA polymerase (Toyobo, Osaka, Japan) and specific primers (Table S2). PCR reaction conditions were as follows: 27 cycles at 94°C for 30 s, 60°C for 30 s, and 74°C for 2 min. PCR products were cloned into the pGEM-T easy cloning vector (Promega, Tokyo, Japan). In order to remove the internal sequence of the target genes, sequences upstream and downstream of the target gene were amplified using pGEM-T easy containing constructed plasmids as the template with specific primers for gene deletion (Table S2). The amplified PCR fragments were excised by *Hind*III digestion and self-ligated. The gene-coding region with a deletion in an internal sequence was

excised by *Eco*RI and inserted into the *Eco*RI site of the suicide vector, pK18mobsacB (20), in order to create the plasmids for gene deletion. Gene-deletion plasmids were conjugated from *Escherichia coli* S17-1 λ pir (22) using StFRB508 mutants as recipients. The StFRB508 recombinants corresponding to single-crossover events were selected on LB agar containing kanamycin and carbenicillin. Deletion mutants of the quorum-sensing genes in StFRB508 were generated by homologous recombination with *sacB* selection. The single-crossover mutants of StFRB508 were streaked onto an LB agar plate with 10% (w/v) sucrose in order to select homologous recombinants. The presence of the expected internal gene deletion in StFRB508 was confirmed by PCR.

Assessment of phenazine production

In the agar plate assay, StFRB508 and its mutants were grown on LB agar plates with or without 1% (w/v) monosaccharides such as D-glucose, D-fructose, D-galactose, L-arabinose, and D-xylose (Kanto Chemical, Tokyo, Japan). A sterile toothpick was dipped into a bacterial suspension and inoculated onto new LB agar prepared in 24-well plates. After an incubation at 30°C for 2 d, phenazine production was identified by the presence of orange pigments. Phenazine production was categorized into three levels: (i) high production (deep orange with diffusion), (ii) low production (moderate orange without diffusion), and (iii) no production (white or very pale yellow).

Phenazine production was also evaluated by the broth assay as described previously with modifications (13). StFRB508 and its mutants were grown for 15 h, inoculated into fresh LB medium (1% inoculum) with or without AHLs, and further incubated for 18 h. One milliliter of each culture was placed in a 1.5-mL microtube. After centrifugation for 5 min, the concentration of phenazine in the culture supernatant was evaluated by measuring absorbance at 365 nm (A_{365}). The turbidity of the culture suspension was assessed by measuring optical density at 600 nm (OD_{600}). Phenazine production was evaluated as relative production (A_{365}/OD_{600}), the maximum value of which equaled 100%.

Extraction and identification of AHLs

StFRB508 and its mutants were inoculated into 4 mL of LB medium containing 1% (w/v) glucose and incubated at 30°C for 18 h under shaking conditions at 150 rpm. Full-grown cultures (500 µL) were transferred into 50 mL fresh medium and incubated at 30°C for 24 h. Cells were removed by centrifugation at 10,000×g for 5 min. Culture supernatants were concentrated by evaporation using a rotary evaporator. They were then extracted with a 3-fold volume of ethyl acetate in a separation funnel. The extract was evaporated to dryness using a rotary evaporator and then dissolved in 500 µL dimethylsulfoxide. The chemical structures of extracted AHLs were analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) as described previously (15).

Quantification of AHLs by LC-MS/MS

The quantification of AHLs was performed using a triple quadrupole/linear ion trap instrument (LIT) (QTRAP5500; AB SCIEX, Tokyo, Japan) with an electrospray ionization (ESI) source coupled to a UHPLC system (Nexera X2; Shimadzu, Kyoto, Japan) in the multiple reaction monitoring (MRM) analysis mode. Chromatographic separation and MS/MS spectra were recorded as reported previously (15). An MRM analysis was performed at 15 V collision energies for the transitions of *m/z* 200→102 for C6-HSL, 214→102 for 3-oxo-C6-HSL, 216→102 for 3-OH-C6-HSL, 244→102 for 3-OH-C8-HSL, 272→102 for 3-OH-C10-HSL, and 300→102 for 3-OH-C12-HSL. Data acquisition and analyses were performed with Multi Quant software ver. 3.0.1 (AB SCIEX). The quantification of AHLs was conducted using synthetic standards. Exudate samples were dissolved in 50% aqueous acetonitrile and filtered through a spin column (Ultrafree-MC 0.45 µm filter unit; Millipore, Bedford, MA). An aliquot of filtered 50% aqueous acetonitrile sample solutions was diluted with a volume of either pure 50% acetonitrile or 50%

Table 1. Suppression of *Pythium*-induced damping-off and root rot in cucumber by *Pseudomonas chlororaphis* subsp. *aurantiaca* StFRB508 and 508ΔPACI.

Bacterial strain added ^a	<i>Pythium</i> added ^a	Surviving plants per flask (%) ^b
None	–	100 a
StFRB508	–	100 a
508ΔPACI	–	100 a
None	+	21 c
StFRB508	+	63 b
508ΔPACI	+	25 c

^a The *Pseudomonas* strain was added at 2×10^7 CFU g⁻¹ soil to 100-mL flasks (50 mL of soil per flask), after planting three 48-h-old, sterile-grown cucumber seedlings per flask. *P. ultimum* was added as a millet-seed inoculum at 2.5 g kg⁻¹ of vermiculite before planting. Plants were harvested after 7 d.

^b Data represent the average of 10 replicates (flasks containing three cucumber plants) per treatment without *P. ultimum* and 16 replicates per treatment with *P. ultimum*. Means within the same column followed by different letters (a–c) are significantly different ($P < 0.05$) according to Tukey's HSD test.

acetonitrile containing known amounts of the AHL standards. The increase in the peak area on the chromatogram corresponded to the amounts of the AHL standards added, thereby enabling the amounts of AHLs in the samples to be estimated. Results were reproduced in three repeated experiments.

Plant disease suppression assays

The biocontrol activity of each strain was evaluated as described previously (23), with slight modifications. Flasks containing 20 g of a 2:1 mixture of vermiculite and peat moss were planted with three cucumber seedlings each and treated with *Pythium ultimum* MAFF425494. StFRB508 mutants were added to soil as a suspension (4 mL in each flask) of cells washed twice in sterile distilled water to give 2×10^7 CFU g⁻¹ soil. Control flasks received the same amount of sterile water. Seedlings were covered with 5 g of untreated soil and flasks were sealed with aerated silicon caps. Microcosms were incubated in a growth chamber at 60% relative humidity and 26°C with light for 16 h, followed by an 8-h dark period. No watering was necessary. After a 7-d incubation, biocontrol activity was assessed by counting the surviving plants in each flask. Data in Table 1 represent means from two individual repetitions of the same experiment. Data from both experiments were analyzed for a trial by a treatment interaction using an analysis of variance, which indicated that data from the two independent trials may be pooled. Means were separated using Tukey's honest significant difference test (at $P \leq 0.05$). Statistical analyses were performed using R version 3.0.1 (<http://www.r-project.org/>).

Nucleotide sequence accession number

The complete genome sequence of *P. chlororaphis* subsp. *aurantiaca* StFRB508 has been deposited in the DDBJ/ENA/GenBank databases under accession no. AP014623.

Results and Discussion

Complete genome sequencing of StFRB508

We previously reported the incomplete draft genome sequence of StFRB508 (13). Two pairs of *luxI* and *luxR* homologues, designated as *phzIR* and *aurIR*, were detected in the draft genome of StFRB508. However, the draft genome sequence of StFRB508 still contains numerous long sequencing gaps. In order to obtain the complete genome sequence of StFRB508, 896,121 paired-end reads with an average read length of 154 bases were re-assembled by applying Eurofins *in silico* Gap Closure pipeline with manual inspection.

Consequently, reads were assembled into 34 large contigs ($>2,000$ bp). The results of PCR for closing assembly gaps revealed that the complete genome of StFRB508 comprised a single circular chromosome of 6,997,933 bp with an average G+C content of 62.82% (Fig. S1). The genome contained 6,309 protein-coding, 16 rRNA, and 67 tRNA genes. In order to elucidate complete quorum-sensing gene networks, we searched for additional AHL-synthase gene homologues in the complete genome. One predicted an ORF (PCAU_2446), which encoded 219 amino acids, showing high identity (more than 94%) to the AHL synthase CsaI from *P. chlororaphis* subsp. *aureofaciens* 30-84 (27). A putative *luxR* homologous gene, designated as *csaR* (PCAU_2447), was mapped upstream of *csaI* as well as 30-84. Furthermore, a previous study reported that HdtS from *Pseudomonas fluorescens* F113 is a third protein family capable of AHL biosynthesis (9). The *hdtS* homologue was also detected in the complete genome of StFRB508 (PCAU_6164). The complete genome sequence of another *P. chlororaphis* subsp. *aurantiaca* strain JD37 has also been described (6). Therefore, we searched quorum sensing-related genes in the complete genome of JD37. Based on the results obtained, *phzIR*, *aurIR*, *csaIR*, and *hdtS* genes were found to be shared between StFRB508 and JD37. These results demonstrate that these quorum-sensing genes are conserved in *P. chlororaphis* subsp. *aurantiaca*.

Quorum sensing-regulated phenazine biosynthesis in StFRB508

In order to elucidate the relationship between quorum sensing-related genes and phenazine production, we constructed multiple gene deletion mutants using a non-marker mutagenesis technique. Our previous findings revealed that phenazine production was not detected in the culture supernatant of the *phzI aurI* double mutant (13). In the present study, we examined phenazine production using the non-marker *phzI aurI* double mutant (508ΔPAI) on LB agar. Similar to our previous findings, no significant amount of phenazine production was observed in 508ΔPAI (Fig. 1A). With respect to growth conditions that favor phenazine production, Yuan *et al.* demonstrated that glucose supplementation enhanced phenazine production in *Pseudomonas* sp. M-18Q (26). In order to investigate the effects of monosaccharides on phenazine production by 508ΔPAI, glucose, fructose, galactose, arabinose, and xylose were added to LB agar at a concentration of 1% (w/v). 508ΔPAI showed low-level phenazine production on LB agar containing glucose, suggesting that the double mutation in *phzI* and *aurI* was not sufficient to abolish phenazine production. This effect was not observed with the addition of other monosaccharides (Fig. 1A).

We then examined phenazine production by all constructed mutants on LB agar containing glucose (Fig. 1B). In cases of AHL-synthase gene mutants, mutations in *aurI* and *csaI* did not reduce phenazine production. The mutation in *phzI* induced a slight reduction in phenazine production. The triple mutant of *phzI*, *aurI*, and *csaI* (508ΔPACI) did not produce phenazine, which was similar to 508ΔPZ as a negative control. The AHLs produced by PhzI were assumed to strongly stimulate phenazine production, whereas those produced by AurI and CsaI only yielded a slight stimulation. Although *Pseudomonas* sp. CMR12a and *P. chlororaphis* subsp. *aureofaciens* 30-84 have a second quorum-sensing system in

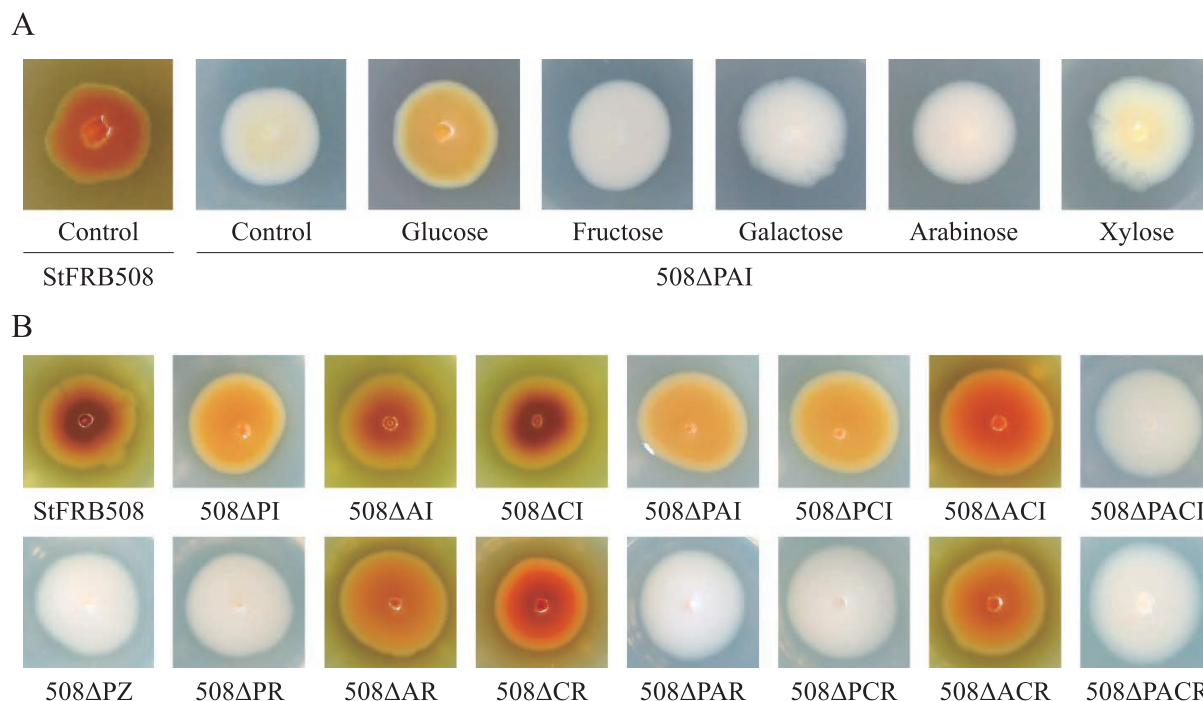


Fig. 1. Phenazine production in *StFRB508* and its mutants when grown on LB agar plates. (A) The *508ΔPAI* mutant strain was inoculated on LB agar containing 1% (w/v) glucose, fructose, galactose, arabinose, and xylose. *StFRB508* and *508ΔPAI* were inoculated on LB agar without mono-saccharides as a control. (B) A series of mutants of *StFRB508* were inoculated on LB agar containing 1% (w/v) glucose. After an incubation at 30°C for 2 d, phenazine production was identified by the presence of orange pigments.

addition to the *phzI/phzR* system, phenazine production was only regulated by the AHLs produced by PhzI (5, 27). This is the first study to show the cross-regulation of phenazine production by AHLs produced by the three independent AHL synthases. In the case of AHL-receptor gene mutants, mutations in *aurR* and *csaR* did not affect phenazine production. However, a single mutation in *phzR* completely diminished phenazine production by *StFRB508*. These results suggest that the expression of the phenazine biosynthesis gene cluster is regulated by the PhzR receptor alone. A previous study reported that the *csaI/csaR* quorum-sensing system regulates the cell surface properties of *P. chlororaphis* subsp. *aureofaciens* 30-84 (27). However, visual differences in colony morphologies were not confirmed between *StFRB508* and mutants of the *aurI/aurR* or *csaI/csaR* quorum-sensing system.

Identification of the structures of AHLs produced by *StFRB508* and its mutants

We previously identified the structures of AHLs produced by PhzI and AurI using a TLC analysis combined with those produced by AHL-reporter strains (13). Our previous findings demonstrated that PhzI catalyzed the biosynthesis of 3-OH-C6-HSL, while AurI catalyzed that of C4-HSL and C6-HSL. However, the use of a TLC analysis to detect the structure of AHL is too simple, and difficulties are associated with elucidating specific structures or small amounts of AHLs. Therefore, in the present study, the chemical structures of AHLs extracted from *StFRB508* and its mutants were analyzed by LC-MS/MS. The mass spectra of AHLs extracted from *StFRB508* culture supernatants and AHL standards are shown in Fig. 2. Six AHLs—C6-HSL, 3-oxo-C6-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C10-HSL,

and 3-OH-C12-HSL—were detected in the *StFRB508* culture supernatant. In order to elucidate the AHL structures produced by three AHL-synthase mutants, the culture supernatants of *508ΔACI* (including the *phzI* gene only), *508ΔPCI* (including the *aurI* gene only), and *508ΔPAI* (including the *csaI* gene only) were prepared and assayed using LC-MS/MS. The culture extract of *508ΔACI* contained four AHLs, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C10-HSL, and 3-OH-C12-HSL (Fig. S2). The culture supernatants of *508ΔPCI* and *508ΔPAI* contained two AHLs, C6-HSL and 3-oxo-C6-HSL (Fig. S3 and 4). We previously demonstrated using a TLC analysis that AurI produced C4-HSL instead of 3-oxo-C6-HSL (13). C4-HSL has also been detected in the culture supernatants of other *P. chlororaphis* strains using a TLC analysis (4, 10). Since the R_f values of C4-HSL and 3-oxo-C6-HSL were similar, we assumed that C4-HSL and 3-oxo-C6-HSL were not separated clearly by TLC. The *csaI/csaR* quorum-sensing system was reported for the first time from *P. chlororaphis* subsp. *aureofaciens* 30-84 (27). However, the structures of AHLs produced by CsaI were not elucidated in that study. As described above, CsaI from *StFRB508* showed greater similarity to that from 30-84. Therefore, CsaI from *Pseudomonas chlororaphis* strains was assumed to commonly produce C6-HSL and 3-oxo-C6-HSL.

Effects of AHL structures on phenazine production in AHL-deficient mutants

AHLs produced by wild-type *StFRB508* were extracted after a 24-h cultivation and quantified by the LC-MS/MS system (Table 2). *StFRB508* mainly produced 3-hydroxy-substituted AHLs, and 3-OH-C6-HSL showed the highest concentration (approximately 6 μ M) among the six AHLs

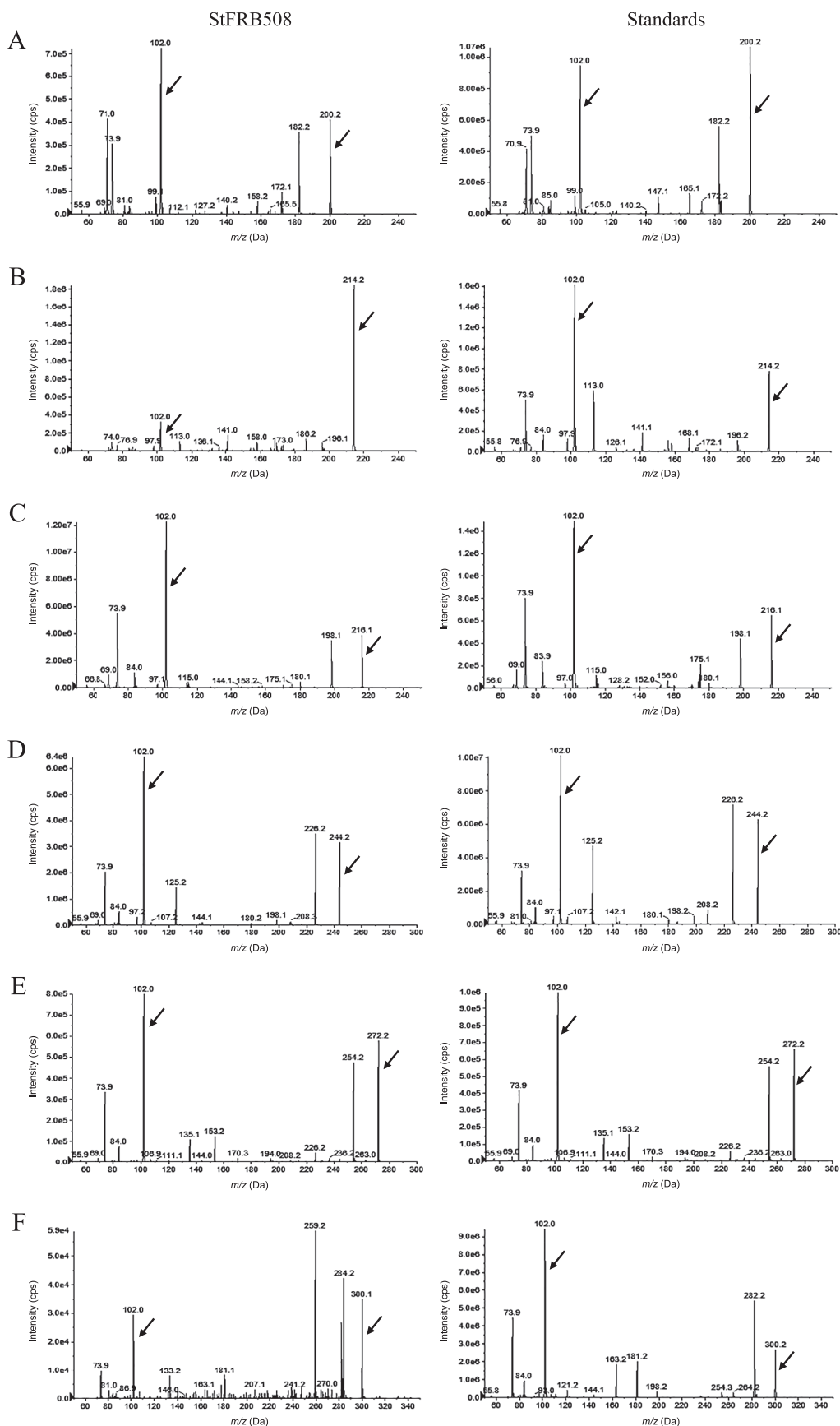
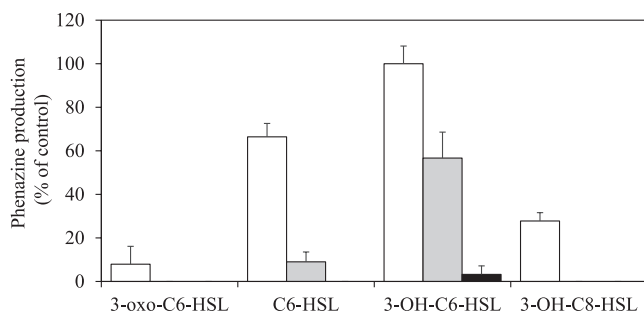


Fig. 2. Mass spectra of AHLs extracted from the cell-free supernatant of *StFRB508* (left panel) and AHL standards (right panel). After fractionation by reverse-phase HPLC, the ESI-MS/MS fragment peaks of AHLs were analyzed. All corresponding peaks for respective C6-HSL (A; m/z 200), 3-oxo-C6-HSL (B; m/z 214), 3-OH-C6-HSL (C; m/z 216), 3-OH-C8-HSL (D; m/z 244), 3-OH-C10-HSL (E; m/z 272), and 3-OH-C12-HSL (F; m/z 300) along with the product ion peaks (m/z 102) are marked by arrows.

Table 2. Quantification of AHLs produced by StFRB508 and its mutants.

Strains	StFRB508	508 Δ ACI	508 Δ PCI	508 Δ PAI	508 Δ PACI
Functional AHL synthase	PhzI, Aurl, CsaI	PhzI	Aurl	CsaI	None
C6-HSL	218 $\pm$ 43 ^a	N.D. ^b	371 $\pm$ 86	4177 $\pm$ 28	N.D.
3-oxo-C6-HSL	159 $\pm$ 67	N.D.	3205 $\pm$ 692	426 $\pm$ 235	N.D.
3-OH-C6-HSL	6006 $\pm$ 713	6653 $\pm$ 2506	N.D.	N.D.	N.D.
3-OH-C8-HSL	1282 $\pm$ 412	1946 $\pm$ 874	N.D.	N.D.	N.D.
3-OH-C10-HSL	768 $\pm$ 210	809 $\pm$ 413	N.D.	N.D.	8 $\pm$ 12
3-OH-C12-HSL	2 $\pm$ 0	3 $\pm$ 1	N.D.	N.D.	N.D.

^a AHL concentrations are expressed in nM.^b N.D., not detected.**Fig. 3.** Phenazine production by 508 Δ PACI in LB medium containing AHLs at concentrations of 0.1 (black bars), 1 (gray bars), and 10 μ M (white bars). Relative phenazine production was evaluated using the value of A_{365}/OD_{600} ; the maximum value obtained for 10 μ M 3-OH-C6-HSL was set as 100%. Results were reproduced in three repeated experiments, and error bars indicate standard deviations.

produced. The concentration and composition ratio of 3-hydroxy-substituted AHLs in 508 Δ ACI (for PhzI) were similar to those of wild-type StFRB508. In contrast, 508 Δ PCI (for Aurl) and 508 Δ PAI (for CsaI) produced higher concentrations of C6-HSL and 3-oxo-C6-HSL than StFRB508. Since AHLs are produced by AHL synthase from *S*-adenosyl-L-methionine (SAM) and an acylated acyl carrier protein (16), the inactivation of PhzI caused an excessive supply of SAM toward Aurl or CsaI in 508 Δ PCI and 508 Δ PAI. Although Aurl and CsaI both produce C6-HSL and 3-oxo-C6-HSL, the composition ratio of the two AHLs is opposite in the products of Aurl and CsaI.

In order to evaluate the effects of AHLs produced by StFRB508 on phenazine production, 508 Δ PACI was inoculated into LB medium containing AHLs at a final concentration of 0.1, 1, or 10 μ M. The results of the assay for phenazine production are shown in Fig. 3. 3-OH-C6-HSL exerted the strongest effects on phenazine production and also stimulated it at the lowest concentration tested (0.1 μ M). Although C6-HSL induced a certain amount of phenazine production at a concentration of 1 μ M, this induction level was 6-fold lower than that of 3-OH-C6-HSL. 3-oxo-C6-HSL and 3-OH-C8-HSL slightly induced phenazine production at a concentration of 10 μ M. Neither 3-OH-C12-HSL nor 3-OH-C10-HSL stimulated phenazine production at the concentrations tested (data not shown). The null mutant of three AHL synthase genes, 508 Δ PACI, produced a small amount of 3-OH-C10-HSL (Table 1). A complete genome analysis of StFRB508 revealed the presence of the *hdtS* gene homologue, which encodes a third class of AHL synthase. *E. coli* harboring *hdtS* from *P. fluorescens* F113 is capable of synthesizing three

kinds of AHLs (9). The small amount of 3-OH-C10-HSL detected in the AHL extracts of 508 Δ PACI was assumed to be produced by HdtS. However, since phenazine production was not stimulated in the presence of 3-OH-C10-HSL, the expression of the phenazine biosynthesis gene cluster may not be practically affected by 3-OH-C10-HSL.

A quorum-sensing mutant exhibited decreased biocontrol efficacy

In our previous study, growth inhibitory activity against the phytopathogenic fungus, *F. oxysporum*, was attenuated in a phenazine-deficient mutant of StFRB508 in *in vitro* antagonism tests (13). In order to evaluate the contribution of AHL production to the *in vivo* biocontrol capacity of StFRB508, we used a cucumber-*P. ultimum* pathosystem. The addition of strain StFRB508 to pathogen-infested soil increased plant survival by approximately 3-fold, which confirmed this strain as an effective biocontrol agent (Table 2). In contrast, the AHL-deficient mutant, 508 Δ PACI, had a significantly impaired capacity to protect cucumber from disease symptoms. In the case of *Pseudomonas* sp. CMR12a, the quorum-sensing mutant deficient in phenazine production had a significantly lower biocontrol capacity against *Rhizoctonia* root rot on bean plants than the wild-type (5). Our results also demonstrated that biocontrol activity through phenazine production was regulated by AHL-mediated quorum sensing in StFRB508. Although phenazine production is considered to be a major factor endowing this strain with plant protection efficacy, its relevance to other phenotypes such as bacterial motility and colonization needs to be established in future studies.

Concluding Remarks

Based on the present results, we propose a mechanism for the regulation of phenazine production by AHL-mediated quorum sensing in StFRB508 (Fig. 4). The results of the AHL quantification demonstrated that of all AHLs, 3-OH-C6-HSL, which exhibited the strongest activity for activating the phenazine biosynthesis gene cluster, was produced by PhzI at the highest concentration in StFRB508. In addition, 3-oxo-C6-HSL and C6-HSL, which were produced by Aurl and CsaI, induced only the slight activation of the gene cluster in parallel with *phzI/phzR* system-mediated regulation. Although Aurl and CsaI do not function as response regulators of the phenazine biosynthetic gene cluster, AHLs produced by Aurl and CsaI exerted heterologous effects on the unrelated *phzI/phzR* quorum-sensing system. The true target genes of the *aurI/aurR* and *csaI/csaR* quorum-sensing systems have not yet been elucidated. Regarding phenazine production, unex-

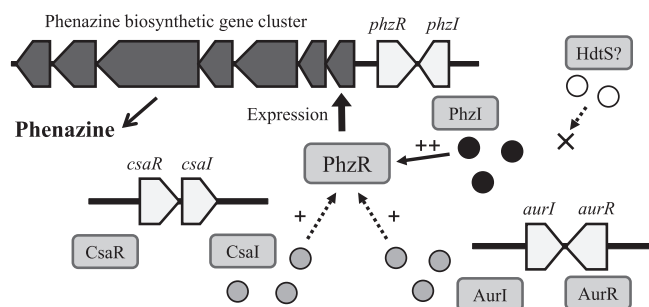


Fig. 4. Schematic representation of the phenazine biosynthetic gene cluster by quorum sensing in StFRB508. Pentagons show the orientation and size of the genes. AHLs produced by *PhzI* (black circles) or *AurI/CsaI* (gray circles), strongly (++) or weakly (+) bind to the *PhzR* receptor protein, respectively. The *PhzR*-AHL complex binds to the promoter and induces the expression of the phenazine biosynthetic gene. The putative AHL products by *HdtS* (open circles) do not bind to the *PhzR* receptor.

pected spontaneous mutations in up to two AHL-synthase genes may not affect phenazine production. AHLs produced by *AurI* and *CsaI* may function as backup signals in the *phzI/phzR* quorum-sensing system in StFRB508.

Acknowledgements

We gratefully thank Dr. Satoshi Ito and Mr. Muneki Ichikawa from Utsunomiya University for synthesizing AHL standards. We thank Ms. Nanase Ichimura from Utsunomiya University for her technical assistance. This work was supported by JSPS KAKENHI Grant Number JP16K07656.

References

- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
- Chhabra, S.R., C. Harty, D.S. Hooi, M. Daykin, P. Williams, G. Telford, D.I. Pritchard, and B.W. Bycroft. 2003. Synthetic analogues of the bacterial signal (quorum sensing) molecule *N*-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. *J. Med. Chem.* 46:97–104.
- Chin-A-Woeng, T.F., G.V. Bloemberg, A.J. van der Bij, K.M. van der Drift, J. Schripsema, B. Kroon, R.J. Scheffer, C. Keel, P.A. Bakker, and H.-V. Tichy. 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol. Plant-Microbe Interact.* 11:1069–1077.
- Chin-A-Woeng, T.F., D. van den Broek, G. de Voer, K.M. van der Drift, S. Tuinman, J.E. Thomas-Oates, B.J. Lugtenberg, and G.V. Bloemberg. 2001. Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Mol. Plant-Microbe Interact.* 14:969–979.
- De Maeyer, K., J. D'Aes, G.K. Hua, M. Perneel, L. Vanhaecke, H. Noppe, and M. Hofte. 2011. *N*-Acylhomoserine lactone quorum-sensing signalling in antagonistic phenazine-producing *Pseudomonas* isolates from the red cocoyam rhizosphere. *Microbiology* 157:459–472.
- Jiang, Q., J. Xiao, C. Zhou, Y. Mu, B. Xu, Q. He, and M. Xiao. 2014. Complete genome sequence of the plant growth-promoting rhizobacterium *Pseudomonas aurantiaca* strain JD37. *J. Biotechnol.* 192:85–86.
- Khan, S.R., J. Herman, J. Krank, N.J. Serkova, M.E. Churchill, H. Suga, and S.K. Farrand. 2007. *N*-(3-hydroxyhexanoyl)-L-homoserine lactone is the biologically relevant quorumone that regulates the *phz* operon of *Pseudomonas chlororaphis* strain 30-84. *Appl. Environ. Microbiol.* 73:7443–7455.
- Lagesen, K., P. Hallin, E.A. Rodland, H.H. Staerfeldt, T. Rognes, and D.W. Ussery. 2007. RNAMmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35:3100–3108.
- Laue, B.E., Y. Jiang, S.R. Chhabra, S. Jacob, G.S. Stewart, A. Hardman, J.A. Downie, F. O'Gara, and P. Williams. 2000. The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via *HdtS*, a putative novel *N*-acylhomoserine lactone synthase. *Microbiology* 146:2469–2480.
- Liu, H., Y. He, H. Jiang, H. Peng, X. Huang, X. Zhang, L.S. Thomashow, and Y. Xu. 2007. Characterization of a phenazine-producing strain *Pseudomonas chlororaphis* GP72 with broad-spectrum antifungal activity from green pepper rhizosphere. *Curr. Microbiol.* 54:302–306.
- Lowe, T.M., and S.R. Eddy. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25:955–964.
- Mavrodí, D.V., W. Blankenfeldt, and L.S. Thomashow. 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu. Rev. Phytopathol.* 44:417–445.
- Morohoshi, T., W.Z. Wang, T. Suto, Y. Saito, S. Ito, N. Someya, and T. Ikeda. 2013. Phenazine antibiotic production and antifungal activity are regulated by multiple quorum-sensing systems in *Pseudomonas chlororaphis* subsp. *aurantiaca* StFRB508. *J. Biosci. Bioeng.* 116:580–584.
- Noguchi, H., T. Taniguchi, and T. Itoh. 2008. MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. *DNA Res.* 15:387–396.
- Okutsu, N., T. Morohoshi, X. Xie, N. Kato, and T. Ikeda. 2015. Characterization of *N*-acylhomoserine lactones produced by bacteria isolated from industrial cooling water systems. *Sensors* 16:44.
- Parsek, M.R., and E.P. Greenberg. 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. U.S.A.* 97:8789–8793.
- Pierson III, L.S., D.W. Wood, and E.A. Pierson. 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu. Rev. Phytopathol.* 36:207–225.
- Pierson III, L.S., and E.A. Pierson. 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl. Microbiol. Biotechnol.* 86:1659–1670.
- Pomini, A.M., P.L. Cruz, C. Gai, W.L. Araujo, and A.J. Marsaioli. 2009. Long-chain acyl-homoserine lactones from *Methylobacterium mesophilicum*: synthesis and absolute configuration. *J. Nat. Prod.* 72:2125–2129.
- Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69–73.
- Schmidt, S., J.F. Blom, J. Pernthaler, G. Berg, A. Baldwin, E. Mahenthiralingam, and L. Eberl. 2009. Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex. *Environ. Microbiol.* 11:1422–1437.
- Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 1:784–791.
- Takeuchi, K., K. Yamada, and D. Haas. 2012. ppGpp controlled by the Gac/Rsm regulatory pathway sustains biocontrol activity in *Pseudomonas fluorescens* CHA0. *Mol. Plant-Microbe Interact.* 25:1440–1449.
- Tambong, J.T., and M. Höfte. 2001. Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. *Eur. J. Plant Pathol.* 107:511–521.
- Thomson, N., M. Crow, S. McGowan, A. Cox, and G. Salmond. 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Mol. Microbiol.* 36:539–556.
- Yuan, L.-L., Y.-Q. Li, Y. Wang, X.-H. Zhang, and Y.-Q. Xu. 2008. Optimization of critical medium components using response surface methodology for phenazine-1-carboxylic acid production by *Pseudomonas* sp. M-18Q. *J. Biosci. Bioeng.* 105:232–237.
- Zhang, Z., and L.S. Pierson III. 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* 67:4305–4315.