Title: Quorum-dependent expression of rsmX and rsmY, small non-coding RNAs, in Pseudomonas syringae

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Pseudomonas syringae pathovars are known to produce N-acyl-homoserine lactones (AHL) as quorum-sensing molecules. However, many isolates, including P. syringae pv. tomato DC3000 (PtoDC3000), do not produce them. In P. syringae, psyI, which encodes an AHL synthase, and psyR, which encodes the transcription factor PsyR required for activation of psyI, are convergently transcribed. In P. amygdali pv. tabaci 6605 (Pta6605), there is one nucleotide between the stop codons of both psyI and psyR. However, the canonical stop codon for psyI in PtoDC3000 was converted to the cysteine codon by one nucleotide deletion, and 23 additional amino acids extended it to a C-terminal end. This resulted in overlapping of the open reading frame (ORF) for psyI and psyR. On the other hand, stop codons in the psyR ORF of P. syringae 7 isolates, including pv. phaseolicola and pv. glycinea, were found. These results indicate that many pathovars of P. syringae have genetically lost AHL production ability by the mutation of their responsible genes. To examine whether PtoDC3000 modulates the gene expression profile in a population-dependent manner, we carried out microarray analysis using RNAs prepared from low- and high-density cells. We found the expressions of rsmX and rsmY remarkably activated in high-density cells. The activated expressions of rsmX and rsmY were confirmed by Northern blot hybridization, but these expressions were abolished in a ΔgacA mutant of Pta6605. These results indicate that regardless of the ability to produce AHL, P. syringae regulates expression of the small noncoding RNAs rsmX/Y by currently unknown quorum-sensing molecules.
1. Introduction

Quorum sensing (QS) is a well-understood mechanism of bacterial cell-cell communication and allows triggering of widespread changes of gene expression in members of the population in a coordinated manner (von Bodman et al., 2003; Ham, 2013; Schuster et al., 2013). QS is mediated by different types of small diffusible molecules, the so-called autoinducers such as N-acyl homoserine lactones (AHLs), fatty acid and butyrolactone derivatives, and a variety of peptide structures. Among them, AHLs are the major autoinducers and used by many bacterial species such as the genera *Erwinia*, *Vibrio*, *Pantoea*, *Rhizobium*, and *Pseudomonas* (von Bodman et al., 2003; Ham, 2013; Schuster et al., 2013).

Although N-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) and N-hexanoyl-L-homoserine lactone (HHL) are known to be major QS molecules in *Pseudomonas syringae*, AHL was not detected in many isolates of *P. syringae* (Cha et al., 1998; Elasri et al., 2001). It is not clear why *P. syringae* has AHL-producing and -lacking isolates, and whether AHL-defective isolates of *P. syringae* produce QS molecules besides AHL. In this study, we investigated AHL production and the structure of *psyI*, a gene encoding AHL synthase, and *psyR*, a gene encoding the QS transcription factor, in *P. syringae* pathovars. The AHL synthase gene *psyI* and AHL transcription factor gene *psyR* are also called *ahlI* and *ahlR* in *P. syringae pv. syringae* B728a (Quiñones et al., 2004) and *psmI* and *psmR* in *P. syringae pv. maculicola* CFBP 10912-9 (Elasri et al., 2001). However, in this paper we used the gene names *psyI* and *psyR* for all AHL synthase genes and transcription factor genes to avoid unnecessary confusion. We found that many isolates, including *Pto DC3000*, do not produce AHLs. Furthermore, mutations of *psyI* and *psyR* are found in many isolates of *P. syringae* that do not produce AHL. These results indicate that some *P. syringae*, including *Pto DC3000*, have genetically lost the ability to produce AHL due to mutation of the corresponding genes.

To examine whether *PtoDC3000* modulates gene expression profiles in a population-dependent manner, we carried out microarray analysis using RNAs prepared from low- and high-density cells. Most upregulated genes in high-density cells contain *rsmX1* to *rsmX5*, *rsmY*, and *rsmZ* genes. The *rsmX*, *rsmY*, and *rsmZ* are major members of small non-coding regulatory RNAs (sRNAs), and are found in *PtoDC3000* (Moll et al., 2010). In *PtoDC3000* *rsmX*, *rsmY*, and *rsmZ* are 112 to 120, 126, and 132 nucleotides in size, respectively (Moll et al., 2010). Small non-coding regulatory RNAs are important components of many physiological and adaptive responses in bacteria (Lapouge et al., 2008; Harfouche et al. 2015). The regulatory mechanisms of small non-coding RNAs were intensively investigated in the biocontrol bacterium *Pseudomonas protegens* CHA0 and the animal pathogen *P. aeruginosa* (Lapouge et al., 2008; Harfouche et al., 2015). In *P. protegens* CHA0, small non-coding RNAs, *rsmX* and *rsmY* express cell density-dependent manner, and capture the translation repressor proteins such as RsmA and RsmE to derepress translation of target mRNAs involved in secondary metabolism and extracellular enzymes. (Kay et al. 2005; Valverde et al. 2004; Lapouge et al., 2008). It is reported that the expression of *rsmX* and *rsmY* in *P. protegens* CHA0 and that of *rsmY* and *rsmZ* in *P. aeruginosa* depend to the GacS/GacA two-component system (Brencic et al. 2009; Humair et al. 2010). Sensor kinase GacS activates and
autophosphorylates by the recognition of yet unidentified signals, and phosphorylates response regulator, GacA.

Upon phosphorylation, GacA activates the transcription of the target genes, *rsmX, rsmY* and *rsmZ* in *P. protegens*. In the promoter of these genes there are conserved sequence elements, the so-called GacA-box or upstream activating sequence (UAS) (Humair et al. 2010). In this study, we found the remarkably upregulated expression of *rsmX* and *rsmY* in high density-cells of *P. syringae*. Based on the evidence, we discuss the involvement of small non-coding RNAs in the system of quorum sensing in *P. syringae*.

**2. Materials and methods**

**2.1. Bacterial strains and growth conditions**

The bacterial strains used in this study are listed in Table 1. *Pseudomonas amygdali pv. tabaci* 6605 and *P. syringae pv. tomato* DC3000 were maintained in King’s B (KB) medium at 27°C, and *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium. *Chromobacterium violaceum* CV026 was grown at 30°C in LB medium with kanamycin at a final concentration of 50 μg/ml (McClean et al., 1997).

**2.2. Detection of N-acylhomoserine lactones**

Bacterial strains were grown in KB medium with 10 mM MgCl₂ for 24 h at 27°C. AHLs extracted with an equal volume of ethyl acetate were detected using C18 reversed-phase thin layer chromatography (TLC Silica gel 60, Merck, Darmstadt, Germany) and the biosensor *C. violaceum* CV026 (Taguchi et al., 2006).

**2.3. DNA sequence analysis**

DNA sequences for *psyI, psyR*, and *rpoD* were collected from the Pseudomonas Genome DB site (http://www.pseudomonas-syringae.org). The small non-coding RNAs in *Pta6605* were searched using each RNA sequence of *Pto* DC3000.

**2.4. RNA extraction and microarray analysis**

*Pta6605* and *PtoDC3000* were cultured overnight in LB supplemented with 10 mM MgCl₂ at 27°C and harvested and suspended in MMMF medium (10 mM mannitol, 10 mM fructose, 50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, pH 5.7) to an OD₆₀ₒ of 0.01 or 1.0, and further incubated for 3.5 h at 27°C. Bacteria were harvested by centrifugation, then total RNA was extracted using a TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, Tokyo, Japan), and further purified by treatment with RNase free DNase (Takara, Kusatsu, Japan) and extraction with water-saturated acidic phenol. Total RNA (10 µg) was used for microarray analysis by a microarray system of Hokkaido system Science Co. Ltd.
2.5. Northern blot hybridization

RNA electrophoresis was carried out according to the method of Rio et al. (2010), and Northern blot hybridization was carried out as described (Rio, 2014). One or 0.5 µg of total RNA was denatured in formamide gel-loading buffer (95% deionized formamide, 0.025% bromophenol blue (w/v), 0.025% xylene cyanol FF (w/v)), fractionated by electrophoresis on a denaturing 8% polyacrylamide gel containing 8 M urea in 0.5 × TBE buffer (50 mM Tris base, 50 mM boric acid, 1 mM EDTA) and then blotted onto a nylon membrane filter Hybond-N+ (GE Healthcare, Tokyo, Japan) using Trans-Blot Turbo (Bio-Rad, Hercules, CA). Blotted RNA was confirmed by staining with 0.02% methylene blue in 0.3 M sodium acetate. DIG-labeled oligonucleotide probes (Table 2) of *rsmX2* and *rsmY* of *Pto DC3000* and *Pta 6605* were prepared using terminal deoxynucleotidyl transferase (Takara) and DIG-11-ddUTP (Sigma-Aldrich, Darmstadt, Germany). Hybridization was performed at 60ºC overnight in a hybridization mix (50% formamide, 5 × SSC, 3 × Denhardt’s solution, 200 µg/mL herring testis carrier DNA, 0.1% SDS) with a DNA probe. Final washes were at 60ºC in a solution containing 0.1 × SSC and 1% SDS. Hybridized RNAs were detected with anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics, Basel, Switzerland), and its chemiluminescent substrate, CDP-star (Thermo Fisher Scientific, Tokyo, Japan). Chemiluminescent was detected using ChemiDoc Touch (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Production of AHL in *Pseudomonas syringae*

AHL production of different pathovars of *P. syringae* was investigated. The isolates investigated are listed in Table 1 and Table S1. Among the isolates of *P. syringae*, AHL production was observed in only *P. amygdali pv. tabaci* 6605 (*Pta 6605*), *pv. tabaci* 11528, and *pv. syringae* B728a, as previously reported (Taguchi et al., 2006; Cheng et al., 2016; Quiñones et al., 2004); however, there are fewer reports of AHL production in other *P. syringae* strains. Using different isolates of *P. syringae* (on a recent taxonomy, they were divided into *P. amygdali*, *P. savastanoi*, and *P. syringae*, Table 1, Gomila et al., 2017), we investigated whether independent isolates produce AHL using a bioassay with *Chromobacterium violaceum* CV026, as shown in Fig. S1. AHL was produced only by *P. amygdali pv. tabaci* 6605, 11528, and *pv. syringae* B728a; the other isolates did not produce detectable AHL.

3.2. Gene structure of *psyI* and *psyR* in *P. syringae*

The *psyI* and *psyR* genes of several isolates of *P. syringae* including genes registered in the *Pseudomonas* database were analyzed (Figs. S2, S3). In *P. syringae*, *psyI* and *psyR* are convergently transcribed, and there is one nucleotide between both stop codons in *Pta 6605*. The DNA sequences for *psyI* and *psyR* are well conserved. However, the canonical stop codon, TGA for *psyI* in *Pto DC3000*, *pv. tomato* T1, and *pv. maculicola* H7608, is converted to the valine codon GTC by one nucleotide deletion, and an additional 22 amino acids
extend to a C-terminal end. This resulted in overlapping of the 3’-end of open reading frames (ORF) for both

\textit{psyI} and \textit{psyR} (Fig. S4). The overlapping structure might interfere with their transcription and translation. On

the other hand, there is a mutational stop codon in the 9th amino acid in \textit{psyR} ORF in \textit{P. savastanoi pv. phaseolicola} (Pph) 1448A, PphY5_2, \textit{pv. maculicola} KN91, \textit{P. amygdali pv. mellea} N6801, and three isolates of \textit{P. savastanoi pv. glycinea} (Fig. S5). Furthermore, three isolates of \textit{P. savastanoi pv. glycinea} have one

nucleotide deletion at 120 nucleotides from the translation start codon, which resulted in a serious frame shift

with additional seven stop codons in their ORFs, and the ORFs are completely destroyed (Fig. S5).

In Fig. 1, we summarized the result of AHL production, schematic depiction of \textit{psyI} and \textit{psyR}, with

phylogenetic analysis of these strains. The phylogenetic tree was generated using the UPGMA method using

\textit{rpoD} sequences by Genetyx version 19.0.0 (Genetyx, Tokyo, Japan). From this result we found that the

mutation of \textit{psyI} or \textit{psyR} occurred in phylogenetically related bacteria, indicating that each isolate of \textit{P. syringae}

has evolved to lose the AHL production.

3.3. Gene expression profiles of low- and high-density cells in \textit{P. syringae pv. tomato} DC3000

To confirm bacterial cell density-dependent gene expression in \textit{PtoDC3000}, we carried out microarray analysis

using RNAs prepared from low- (OD\textsubscript{600} = 0.01) and high-density (OD\textsubscript{600} = 1.0) cells. The result is shown in

Table S2: the expressions of 303 genes were up-regulated (Table S3), and 101 genes were down-regulated in the

high-density cells (Table S4). Among the up-regulated genes, remarkably high expression was observed in small

non-coding regulatory RNAs, i.e., five members of \textit{rsmX} (\textit{rsmX1-X5}), \textit{rsmY}, and \textit{rsmZ} (Fig. 2 and Table 3, Moll

et al., 2010). Expression of \textit{rsmX1-X5} and \textit{rsmY} was increased 9- to 56-fold in high-density cells, whereas that of

\textit{rsmZ} increased 2.6-fold. A significant level of \textit{rsmY} expression was also observed in low-density cells, but it

remarkably upregulated in high-density cells. On the other hand, there are also down-regulated genes in the

high-density condition as indicated blue dots in Fig. 2. There are significant number of flagella-related genes in

the genes which remarkably down-regulated (Table S4), indicating that flagella motility decreases in high-
density condition. However, the relationship of most genes to QS is not clear.

3.4. \textit{rsmX}, \textit{rsmY}, and \textit{rsmZ} genes in \textit{Pta6605}

Each ortholog of \textit{rsmX} (\textit{rsmX1-X5}), \textit{rsmY}, and \textit{rsmZ} was identified in \textit{Pta6605} draft sequences (Fig. S6). The

upstream activating sequences (UAS, Humair et al., 2010) were well conserved in the upstream promoter

regions of five orthologs of \textit{rsmX} and \textit{rsmY}. However, it was less conserved in \textit{rsmZ}. All \textit{rsmX}, \textit{rsmY}, and \textit{rsmZ}

possessed many GGA motifs in the transcribed regions. At the 3’ end of five \textit{rsmX} and \textit{rsmY} genes, there were

sequences to form a stem-loop structure, which functions as a \(\rho\)-independent terminator as found in \textit{PtoDC3000}

(Moll et al., 2010).
3.5. Expression of rsmX2 and rsmY in PtoDC3000 and Pta6605

Enhanced expression of small non-coding RNAs was also investigated by Northern blot hybridization in PtoDC3000 and Pta6605. Total RNAs prepared from low- (OD600 = 0.01) and high-density (OD600 = 1.0) cells of the AHL production–defective bacterium PtoDC3000 wild-type (WT) and the AHL-producing bacterium Pta6605. The microarray results showed that the expression of rsmX2 was the strongest among the rsmX family in the high-density cells. Furthermore, rsmY was the strongest sRNA in high-density cells (Table 3). Therefore, we carried out Northern blot hybridization to detect rsmX2 and rsmY in a low- and high-density cell conditions.

In PtoDC3000, the signal corresponding to rsmX2 was detected in only high-density cells but not in low-density cells (Fig. 3A). The signal for rsmY was also strongly detected in high-density cells but was only weakly detected in low-density cells (Fig. 3B). We also investigated transcripts for rsmX2 and rsmY in Pta6605. The results were almost identical to the case of PtoDC3000: there were almost no signals for rsmX2 and rsmY in low-density cells, whereas significant levels of transcripts for rsmX2 and rsmY were observed in high-density cells (Fig. 3CD).

3.6. Expression of rsmX2 and rsmY in Pta6605 ΔgacA

Because it was reported that the expression of small non-coding RNAs is dependent on the GacS/GacA two-component system in P. fluorescens (P. protegens) CHA0 and P. aeruginosa (Kay et al., 2005, 2006; Valverde et al., 2003), we investigated the expression of rsmX2 and rsmY in Pta6605 using a previously generated ΔgacA mutant (Marutani et al., 2008). As shown in Fig. 4, the expression of rsmX2 was not detected, and that of rsmY was only weakly detected and not significantly increased in high-density cells of the ΔgacA mutant.

3.7. Expression of rsmX2 and rsmY in Pta6605 ΔpsyI, ΔpsyR, and ΔaefR

Because it is known that AHLs are major QS molecules in P. syringae, we investigated the expression of rsmX2 and rsmY in both WT and previously generated QS-defective mutants such as ΔpsyI and ΔpsyR mutant strains of Pta6605 (Taguchi et al., 2006; Ichinose et al., 2018). As shown in Fig. 5, the expressions of rsmX2 and rsmY in these mutant strains were almost identical to those of the WT strain. It is also known that AHL production in the ΔaefR mutant of Pta6605 was also abolished (Kawakita et al., 2012). The expressions of rsmX2 and rsmY were also induced in high-density cells in these mutants. However, the expression of rsmX2 and rsmY in low-density cells was stronger in the ΔaefR mutant than in the WT strain. Furthermore, we investigated the effect of exogenous application of AHL (at 10 µM final concentration of each HHL and OHHL) on the expression of rsmX2 in the AHL-production defective mutant Pta6605 ΔpsyI and PtoDC3000 WT strains. The expression of rsmX2 was evaluated as a β-galactosidase activity which derived from rsmX2 promoter. We found that the β-galactosidase activity derived from rsmX2 promoter increased in a bacterial density-dependent manner and
regardless of the existence of AHL in these strains (data not shown). These results clearly showed that AHL did not affect the expression of rsmX2.

4. Discussion

4.1. Decline in AHL production capacity

Although AHL production was reported previously in some strains of P. syringae (Cha et al., 1998; Elasri et al., 2001), this study revealed that AHL-producing bacteria are not majority. In this study, we investigated the AHL production by biosensor bacteria along with genetic information on AHL synthases (psyI) and AHL transcription factors (psyR) of several isolates of P. syringae. As a result of the investigation, we found that many isolates of P. syringae not only abolished AHL production, but also mutated AHL production-related genes. Interestingly, P. syringae isolates belonging to the same clade have the same or similar gene structures of psyI and psyR (Fig. 1). Each isolate belonging to the same clade as Pph1448A has substituted stop codon at the position of the 9th amino acid of psyR. Furthermore, three isolates of P. savastanoi pv. glycinea in this clade not only have the same substitution at the 9th amino acid, but also one nucleotide deletion with a serious frame shift. Overlapping of ORF for both psyI and psyR occurred in all isolates of the clade to which PtoDC3000 belongs. This suggests that the mutation of psyI and psyR genes occurred with differentiation of P. syringae pathovars. It means that ancestors of P. syringae had produced AHL, but that most P. syringae strains had abolished it because AHL production might become inconvenient for successful infection by the pathogenic bacteria. Thus, most P. syringae might have abandoned production of AHL by the introduction of a mutation in psyI or psyR genes.

4.2. Effect of AHL on plant physiology

Why did many isolates of P. syringae abandon the ability to produce AHL? Although AHLs are a communication tool used by individual bacterial cells to monitor the population density and coordinate gene expression profiles, AHLs are also recognized by plants and animals (Hartmann and Schikora, 2012; Teplitski et al., 2011). Accumulated reports suggest that AHL induces plant growth and plant defense responses (Schenk and Schikora, 2015). The effect of AHLs varies depending on the type of AHL and plant species. However, HHL-treated tomatoes accumulated salicylic acid and activated the transcription of PR-1 and chitinase genes (Schuhegger et al., 2006), suggesting that AHLs are undesired molecules in tomato infection by PtoDC3000. How do plants recognize AHL? In Arabidopsis, OHHL and N-3-oxo-octanoil-homoserine lactone (OOHL) induced root elongation at 1–10 µM concentrations (Liu et al., 2012). In this AHL-mediated elongation of Arabidopsis roots, GCR1, a G-protein-coupled receptor, and GPA1, the sole canonical Ga subunit, are involved (Liu et al., 2012). Furthermore, AHL was amidolyzed by a plant-derived fatty acid amide hydrolase to yield L-homoserine in Arabidopsis (Palmer et al., 2014). The accumulation of L-homoserine promotes plant
growth at low concentrations by stimulating transpiration, while higher concentrations inhibit growth by
stimulating ethylene production (Palmer et al., 2014).

4.3. Gac/Rsm system controls QS-dependent bacterial phenotype

The expressions of *rsmX* and *rsmY* are activated in high density-cells of *PtoDC3000* and *Pta6605* regardless
the production of AHL. The expression of *rsmX* and *rsmY* was investigated using multiple mutant strains of
*Pta6605*. The ∆*gacA* mutant completely abolished the expression of *rsmX*, and that of *rsmY* was remarkably
reduced (Fig. 4). The low level of *rsmY* was expressed regardless of bacterial cell density, indicating that *rsmY*
is under the control of an expression system other than the GacS/A two-component system. The expression of
*rsmX* and *rsmY* was not changed in the ∆*psyI* and ∆*psyR* mutant strains of *Pta6605* (Fig. 5). Furthermore,
exogenous application of AHL in *Pta6605* ∆*psyI* and *PtoDC3000* WT did not affect the expression of *rsmX*
(data not shown). This result indicates that the Rsm-mediated gene expression pathway might control the AHL-
mediated gene expression pathway.

Previously, we investigated the phenotype of a ∆*gacA* mutant strain in *Pta6605* (Marutani et al., 2008).
The ∆*gacA* mutant lost swarming motility and production of fluorescent pigment, and remarkably reduced AHL
production. We speculated that the swarming motility and pigment production were regulated via a Gac/Rsm
pathway because the addition of a mixture of HHL and OHHL to *gac*-defective mutants did not restore these
phenotypes (Marutani et al., 2008). The ∆*gacA* mutant also had reduced expression levels of *algT* and *hrp*
genes, adhesion, and exopolysaccharide production. It is possible that these phenotypes might be also regulated
via a Gac/Rsm signal pathway.

*syringae* B728a and BR2R, and found that *rsmA*-overexpressers abolished production of phytotoxins such as
phaseolotoxin, syringomycin, and tabtoxin. Furthermore, these strains diminished the production of protease
and pyoverdine as well as swarming motility, and remarkably reduced the ability to cause disease in their host
plants (Kong et al., 2012). These results indicated that RsmA repressed the translation of virulence-related
mRNA. In *PtoDC3000*, five members of RsmA/CsrA are known (Ferreiro et al., 2018). Ferreiro et al. (2018)
generated deletion mutants for the most conserved *csrA1*, *csrA2*, and *csrA3*, and investigated the possible
involvement of CsrA1, CsrA2, and CsrA3 in virulence-related traits. Thus, Ferreiro et al. (2018) found that the
∆*csrA3* enhanced alginate production accompanying activation of the alginate biosynthesis gene *algD*,
swarming motility, and *hrp* gene expression, suggesting that CsrA3 plays a pivotal role in bacterial virulence.

Very recently it was reported that motility, expression of type III secretion-related genes, and biofilm
formation were regulated by both the Gac/Rsm regulatory system and cyclic di-guanosine monophosphate (c-di-
GMP) (Bhagirath et al., 2018). High levels of c-di-GMP were reported to correlate with evasion of plant
immunity in *Pseudomonas* by inhibiting flagellin synthesis, although the in planta growth of *PtoDC3000* in
which c-di-GMP is high was drastically reduced after the spray inoculation by impaired migration into the apoplast (Pfeilmeier et al., 2016). Thus, there is a link between c-di-GMP and rsmZ in the regulation of the motile-sessile switch in *P. aeruginosa* and *P. fluorescens* (Petrova et al., 2014). A mutant for GcbA, a diguanylate cyclase (GDC), had enhanced motility but reduced initial surface attachment activity and *rsmZ* expression. On the contrary, a *gcbA*-overexpression strain had reduced motility, but initial surface attachment activity and *rsmZ* expression were activated. Furthermore, changes in the above activities in the ∆*gcbA* mutant were restored by the overexpression of *rsmZ* (Petrova et al., 2014). These results indicate that the functions of GcbA are at least partially dependent on *rsmZ* and that c-di-GMP potentially contributes to the regulation of *rsmZ* abundance (Petrova et al., 2014).

The expressions of *rsmX2* and *rsmY* were cell density–dependent (Fig. 3). However, the signal(s) that activate the Gac two-component system were not clear. Besides AHL, *P. syringae* should produce and secrete novel signal(s) to recognize bacterial population by themselves. Further investigation is necessary.

**Conflicts of Interest:** The authors declare no conflict of interest.

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syringae pv. tabaci regulates virulence factors via \( N \)-acyl homoserine lactone and fatty acid synthesis. J. Bacteriol. 188, 8376-8384.


Figure legends

**Fig. 1.** Structure of quorum sensing genes, *psyI* and *psyR*, and production of N-acylhomoserine lactones in *Pseudomonas syringae* pathovars and isolates.

A phylogenetic tree of pathovars and isolates of *P. syringae* was constructed based on the sequence of *rpoD*. A gene for AHL synthase, *psyI*, and a gene for a transcriptional regulator, *psyR*, are transcribed convergently. Overlapping regions of two arrows indicate overlapping of two open reading frames. The dark portion in arrows of *psyR* indicates an untranslatable sequence by the stop codon(s) generated by the nucleotide substitution(s). The right column of AHL indicates the experimental result of AHL production. AHL detection is indicated as plus (+) and minus (-). NT: not tested.

**Fig. 2** Result of microarray analysis.

The open source R software (R version 3.2.5, [http://www.r-project.org/](http://www.r-project.org/)) was used for microarray analysis and visualization. Genes expressed in *P. syringae* pv. *tomato* DC3000 at high cell density (OD<sub>600</sub> = 1.0) and at low cell density (OD<sub>600</sub> = 0.01) were plotted. Each dot represents individual level of gene expression. Red dots and blue dots indicate the genes expressed more than twice as much and less than half as much in high cell density conditions, respectively, whereas grey dots indicate the genes expressed more than half and less than twice as much. Five *rsmX*, *rsmY* and *rsmZ* genes are shown.

**Fig. 3** Northern blot hybridization of *rsmX2* (A and C) and *rsmY* (B and D) of *Pto*DC3000 (A and B) and *Pta*6605 (C and D).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of *Pto*DC3000 and 0.5 µg of *Pta*6605) prepared from low-density cells (lane L, OD<sub>600</sub> = 0.01) and high-density cells (lane H, OD<sub>600</sub> = 1.0) were used for Northern blot hybridization. DIG-labeled oligonucleotides, *Pto*-rsmX2-R and *Pto*-rsmY-R, were used as hybridization probes for *Pto*DC3000, and *Pta*-rsmX2-R and *Pta*-rsmY-R for *Pta*6605, respectively.

**Fig. 4** Northern blot hybridization of *rsmX2* (A and C) and *rsmY* (B and D) of *Pta*6605 WT (A and B) and *Pta*6605Δ*gacA* (C and D).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of *Pta*6605) prepared from low-density cells (lane L, OD<sub>600</sub> = 0.01) and high-density cells (lane H, OD<sub>600</sub> = 1.0) were used for Northern blot hybridization. DIG-labeled oligonucleotides, *Pta*-rsmX2-R, and *Pta*-rsmY-R were used as hybridization probes for *Pta*6605.
Fig. 5 Northern blot hybridization of rsmX2 (A, C, E and G) and rsmY (B, D, F and H) of Pta6605 WT (A and B), Pta6605∆psyI (C and D), Pta6605∆psyR (E and F), and Pta6605∆aeR (G and H).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of Pta6605) prepared from low-density cells (lane L, OD$_{600}$ = 0.01) and high-density cells (lane H, OD$_{600}$ = 1.0) were used for Northern blot hybridization. DIG-labeled oligonucleotides, Pta-rsmX2-R, and Pta-rsmY-R were used as hybridization probes for Pta6605.

SUPPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Fig. S1 AHL production in different P. syringae isolates.

Ethyl acetate extract from 2 ml of bacterial culture from each bacterium was spotted on TLC plates. Red marks indicate that DNA sequence analysis was also done as shown in Fig. 1.

Fig. S2 Amino acid alignment of PsyI protein of different isolates of P. syringae.

Asterisks and dots below the sequences indicate the same and similar amino acids, respectively.

Fig. S3 Amino acid alignment of PsyR protein of different isolates of P. syringae.

Stop codons are indicated as red asterisks. Asterisks and dots below the sequences indicate the same and similar amino acids, respectively.

Fig. S4 Comparisons between DNA and amino acid sequences of Pta6605 and PtoDC3000.

Both 3’-ends of psyI and psyR and corresponding C-terminal regions of PsyI (red) and PsyR (blue) are shown.

In PtaDC3000, deletion of one nucleotide caused a frame shift that eliminated the stop codon and extended the additional C-terminal sequence. Consequently, 69 bp of both ORF at 3’ ends of psyI and psyR are overlapped in PtoDC3000.

Fig. S5 Comparisons of psyR DNA sequences and PsyR deduced amino acid sequences between Pta6605 (Pta) and P. savastanoi pv. glycinea KN44 (Pgl).

Both psyR DNA sequences are highly homologous each other at 99% identity, and identical nucleotides are indicated as asterisks. The nucleotides and amino acids of Pgl different from Pta is shown in red. Stop codons are also shown as red asterisks.

Fig. S6 DNA sequences of rsmX, rsmY, and rsmZ of Pta6605.

Upstream promoter regions and transcribed regions of five rsmX (A), rsmY (B), and rsmZ (C) are shown. The
consensus upstream activating sequence (UAS) (Humair et al. 2010), -35 and -10 promoter sites are indicated in red. Transcription start sites are indicated by arrows and shown as +1. The GGA motifs in the transcribed region are indicated by blue letters. The sequences highlighted in green are identical in all five rsmX genes and similar to the rsmY gene at their 3’ end. These sequences are predicted to form a stem-loop, which functions as a rho-independent terminator. Underlined regions in rsmX2 and rsmY were used as probes in Northern blot hybridization.

Table S1 Bacterial strains used in AHL assay.

Table S2 Gene expression profiles of high-density cells compared with those of low-density cells in PtoDC3000 by microarray analysis.

Table S3 Genes whose expressions were increased more than 2 times at high cell density than at low cell density.

Table S4 Genes whose expressions were decreased to less than half of the low cell density at high bacterial cell density.
Table 1 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>MAFF number</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. amygdali</em> pv. <em>tabaci</em> isolate 6605</td>
<td>-</td>
<td><em>Pta</em>6605</td>
</tr>
<tr>
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<td>-</td>
<td><em>Pta</em>11528</td>
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<tr>
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<td><em>Pla</em>YM7902</td>
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<td><em>Pme</em>N6801</td>
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<tr>
<td><em>P. amygdali</em> pv. <em>mosprunorum</em> FTRS_U7805</td>
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<td><em>P. savastanoi</em> pv. <em>glycinea</em> BR1</td>
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<td><em>Pg</em>BR1</td>
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<td><em>P. savastanoi</em> pv. <em>glycinea</em> KN44</td>
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<td><em>P. savastanoi</em> pv. <em>phaseolicola</em> 1448A</td>
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Table 2 Oligonucleotides used for Northern blot hybridization

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<tr>
<td>Pto rsmX2-R</td>
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<tr>
<td>Pto rsmY-R</td>
<td>AAAGAAACCGCCTAAGCCTGGGCTTCAGACTTCTCCCTCTGCCTTTCAAGCCGCAGCAGCATCTGC</td>
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<tr>
<td>Pta rsmX2-R</td>
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</tr>
<tr>
<td>Pta rsmY-R</td>
<td>AAAGAAACCGCCTAAGCCTGGGCTTCAGACTTCTCCCTCTGCCTTTCAAGCCGCAGCAGCATCTTC</td>
</tr>
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</table>

Each nucleotide is the complementary sequence of the corresponding small non-coding RNA, and covers 2/3 of the full size RNA.
<table>
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<tr>
<th>Gene name</th>
<th>Product name</th>
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<th>HCD</th>
<th>HCD/LCD</th>
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<td>PSPTO_5652</td>
<td>rsmZ</td>
<td>23.53</td>
<td>61.26</td>
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Fig. 1. Structure of quorum sensing genes, *psyI* and *psyR*, and production of N-acylhomoserine lactones in *Pseudomonas syringae* pathovars and isolates. A phylogenetic tree of pathovars and isolates of *P. syringae* was constructed based on the sequence of *rpoD*. A gene for AHL synthase, *psyI*, and a gene for a transcriptional regulator, *psyR*, are transcribed convergently. Overlapping regions of two arrows indicate overlapping of two open reading frames. The dark portion in arrows of *psyR* indicates an untranslatable sequence by the stop codon(s) generated by the nucleotide substitution(s). The right column of AHL indicates the experimental result of AHL production. AHL detection is indicated as plus (+) and minus (-). NT: not tested.
Fig. 2 Result of microarray analysis.
The open source R software (R version 3.2.5, http://www.r-project.org/) was used for microarray analysis and visualization. Genes expressed in *P. syringae* pv. *tomato* DC3000 at high cell density (OD$_{600}$ = 1.0) and at low cell density (OD$_{600}$ = 0.01) were plotted. Each dot represents individual level of gene expression. Red dots and blue dots indicate the genes expressed more than twice as much and less than half as much in high cell density conditions, respectively, whereas grey dots indicate the genes expressed more than half and less than twice as much. Five *rsmX*, *rsmY* and *rsmZ* genes are shown.
Fig. 3 Northern blot hybridization of *rsmX2* (A and C) and *rsmY* (B and D) of *PtoDC3000* (A and B) and *Pta6605* (C and D).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of *PtoDC3000* and 0.5 µg of *Pta6605*) prepared from low-density cells (lane L, OD$_{600} = 0.01$) and high-density cells (lane H, OD$_{600} = 1.0$) were used for Northern blot hybridization. DIG-labeled oligonucleotides, Pto-*rsmX2*-R and Pto-*rsmY*-R, were used as hybridization probes for *PtoDC3000*, and Pta-*rsmX2*-R and Pta-*rsmY*-R for *Pta6605*, respectively.
Fig. 4 Northern blot hybridization of *rsmX2* (A and C) and *rsmY* (B and D) of *Pta*6605 WT (A and B) and *Pta*6605Δ*gacA* (C and D).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of *Pta*6605) prepared from low-density cells (lane L, OD<sub>600</sub> = 0.01) and high-density cells (lane H, OD<sub>600</sub> = 1.0) were used for Northern blot hybridization. DIG-labeled oligonucleotides, *Pta*-rsmX2-R, and *Pta*-rsmY-R were used as hybridization probes for *Pta*6605.
Fig. 5 Northern blot hybridization of \( rsmX2 \) (A, C, E and G) and \( rsmY \) (B, D, F and H) of \( \text{Pta}6605\text{WT} \) (A and B), \( \text{Pta}6605\Delta\text{psyI} \) (C and D), \( \text{Pta}6605\Delta\text{psyR} \) (E and F), and \( \text{Pta}6605\Delta\text{aefR} \) (G and H).

In each set of experiments, the methylene blue-stained membrane is shown on the left, and the corresponding hybridization result is shown on the right. Total RNAs (1 µg of \( \text{Pta}6605 \)) prepared from low-density cells (lane L, \( \text{OD}_{600} = 0.01 \)) and high-density cells (lane H, \( \text{OD}_{600} = 1.0 \)) were used for Northern blot hybridization. DIG-labeled oligonucleotides, Pta-\( \text{rsm}X2\)-R, and Pta-\( \text{rsm}Y\)-R were used as hybridization probes for \( \text{Pta}6605 \).