Characterization of quorum sensing-controlled transcriptional regulator MarR and Rieske (2Fe–2S) cluster-containing protein (Orf5), which are involved in resistance to environmental stresses in *Pseudomonas syringae* pv. *tabaci* 6605

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SUMMARY

Pseudomonas syringae pv. *tabaci* 6605 (Pta6605) produces acyl homoserine lactones (AHLs), quorum sensing (QS) molecules that are indispensable for virulence in host tobacco infection. Genome-wide transcriptional profiling of several QS-defective mutants revealed that the expression of the genes encoding the MarR family transcriptional regulator (MarR) and a Rieske 2Fe–2S cluster-containing protein (Orf5) located adjacent to *psyl*, a gene encoding AHL synthetase, are significantly repressed. Exogenous application of AHL recovered the expression of both *marR* and *orf5* genes in the Δ *psyl* mutant, indicating that AHL positively regulates the expression of these genes. To investigate the role of these genes in the virulence of Pta6605, Δ *marR* and Δ *orf5* mutants were generated. Both mutants showed decreased swimming and swarming motilities, decreased survival ability under oxidative and nitrosative stresses and, consequently, reduced virulence on host tobacco plants. Transmission electron micrographs showed that the structure of the cell membranes of Δ *marR* and Δ *orf5* mutants was severely damaged. Furthermore, not only the ratio of dead cells, but also the amount of flagella, extracellular DNA and protein released into the culture supernatant, was significantly increased in both mutants, indicating that the disruption of *marR* and *orf5* genes might induce structural changes in the membrane and cell lysis. Because both mutants showed partly similar expression profiles, both gene products might be involved in the same regulatory cascades that are required for QS-dependent survival under environmentally stressed conditions.

Keywords: extracellular DNA, nitrite, Rieske 2Fe–2S cluster, superoxide, surface motility.

INTRODUCTION

The Gram-negative phytopathogenic bacterium *Pseudomonas syringae* pv. *tabaci* 6605 (Pta6605) causes wildfire disease on host tobacco plants (Ichinose *et al.*, 2003; Taguchi *et al.*, 2006b). We have been using this bacterium as a model system to examine bacteria–plant interactions. Previous studies in our laboratory have revealed that a variety of factors contribute to its virulence: flagella-mediated motility (Taguchi *et al.*, 2010), flagellin glycosylation for its stabilization (Taguchi *et al.*, 2006b, 2008), quorum sensing (QS) mediated by acyl homoserine lactones (AHLs) (Taguchi *et al.*, 2006a), a transcriptional regulator, AefR, for the QS system (Kawakita *et al.*, 2012), type III secretion system (T3SS) (Marutani *et al.*, 2005), Gac two-component system (Marutani *et al.*, 2008) and a global transcriptional regulator for virulence, Vfr (Taguchi and Ichinose, 2013).

Phenotypic analysis of flagella-mediated motility-defective mutants has demonstrated that the ability to produce AHL is lost or reduced by lower expression of *psyl* and *psyR* genes encoding the AHL synthesis protein and its regulator PsyR (Kanda *et al.*, 2011; Taguchi *et al.*, 2010). These results indicate that flagellum-dependent motility may be correlated with the AHL-mediated QS system.

Furthermore, our microarray analyses have shown that all AHL production-defective mutant strains exhibit drastically reduced expression of genes encoding the MarR (multiple antibiotic resistance regulator) family transcriptional regulator and a putative Rieske (2Fe–2S) cluster-containing protein (described in the Results section). However, the function of MarR and putative Rieske (2Fe–2S) cluster-containing proteins is not known, although their expression seems to be under the regulation of QS.

Phytopathogenic bacteria need to adapt to a variety of environmental conditions. Members of the MarR family of transcriptional regulators are involved in the regulation of multiple virulence genes, including those important in antibiotic resistance, the environmental stress response and the production of virulence factors

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(Ellison and Miller, 2006; Perera and Grove, 2010). One MarR family transcriptional regulator, SlyA, has also been reported to regulate virulence in several animal (Ellison and Miller, 2006) and phytopathogenic (Haque *et al.*, 2009; Zou *et al.*, 2012) bacteria. In a *slyA* mutant of a phytopathogen *Dickeya dadantii* 3937, the production of pectate lyase, a major virulence determinant and exopolysaccharide, was reduced, and this mutant had an attenuated ability to induce the maceration of potato tubers, indicating that *slyA* plays an important role in the expression of multiple virulence-related genes (Haque *et al.*, 2009).

The simplest Fe–S proteins are the rubredoxins in which Fe is coordinated by four cysteines. The principal basic structure of the Fe–S cluster is 2Fe–2S (Kiley and Beinert, 2003). These Fe–S cluster-containing proteins are called Rieske proteins after their first discoverer (Schneider and Schmidt, 2005). Fe–S proteins play an important role in electron transfer and catalysis of metabolism. Especially in bacteria, Fe–S proteins are key components acting as redox agents in the pathways of photosynthesis and nitrogen fixation (Kiley and Beinert, 2003). The Fe–S centre is involved in electron transfer and is bound to the protein with two cysteine–sulfur atoms and two nitrogen atoms from the imidazole ring of histidine residues present in the conserved sequence, called Rieske motifs (Schmidt and Shaw, 2001). It has been reported that 2Fe–2S cluster-containing proteins possess sensing and transcriptional functions. As a well-known example, SoxR in *Escherichia coli* and *Salmonella* is reported to be both a sensor and a transcriptional factor that responds to environmental oxidative and nitrosative stresses (Shen and Fang, 2012). These free radicals are produced by respiration or as a host defence response.

In this study, we constructed gene-disrupted mutant strains for MarR and a putative Rieske 2Fe–2S cluster-containing protein to elucidate the functions of these gene products in the phytopathogenic bacterium *Pta6605*. Using these mutants, we investigated survival under oxidative and nitrosative stress conditions, the ratio of dead cells in liquid culture, flagella-dependent surface motilities, structure of the cell surface by transmission electron microscopy (TEM), release of extracellular DNA or proteins and virulence to the host tobacco plant. Together with the

results of microarray analysis, we discuss the role of gene expression in the tolerance to environmental stress under regulation by AHL-mediated QS.

RESULTS

Gene expression profiles of AHL production-defective mutants

The results of microarray analyses in several previously characterized AHL production-defective mutants are shown in Table S1 (see Supporting Information). These mutants include a flagellin-defective mutant $\Delta fliC$ (Taguchi *et al.*, 2010), flagella HAP2-defective mutant $\Delta fliD$ (Shimizu *et al.*, 2003), flagellar motor protein-defective mutant $\Delta motABCD$ (Kanda *et al.*, 2011), flagellin glycosyltransferase-defective mutant $\Delta fgt1$ (Taguchi *et al.*, 2010), AHL synthesis protein-defective mutant $\Delta psyI$ (Taguchi *et al.*, 2006a), a defective AHL synthesis regulator mutant $\Delta aefR$ (Kawakita *et al.*, 2012) and a *gac* two-component system-defective mutant $\Delta gacA$ (Marutani *et al.*, 2008). We summarized the expression levels of orthologues of PSPPH_1609 to PSPPH_1615 and PSPPH_1617 in *Pta6605*, when compared with the wild-type (WT), by microarray analysis, as shown in Table 1. The orthologues of PSPPH_1613, PSPPH_1612, PSPPH_1611, PSPPH_1610 and PSPPH_1609 consist of an operon and are tentatively designated as *orf1* to *orf5*, respectively, whereas the orthologues of PSPPH_1614, PSPPH_1615 and PSPPH_1617 are the *psyI*, *psyR* and *marR* genes, respectively (Fig. 1). *orf5* in this gene cluster is predicted to encode a Rieske (2Fe–2S) cluster-containing protein, whereas *orf3* encodes an uncharacterized dihydrodipicolinate synthetase family protein, and *orf1*, *orf2* and *orf4* each encode hypothetical proteins. Among these genes, the expression of *psyI* and *orf1*–*orf5* was almost abolished, whereas that of *psyR* and *marR* was strongly reduced, although weakly expressed.

In this study, we focused on genes encoding the MarR family transcriptional regulator and Rieske (2Fe–2S) cluster-containing protein to assess their significance in the virulence of *Pta6605*.

Table 1 Gene expression profiles of acyl homoserine lactone (AHL) production-defective mutants compared with those of the wild-type (WT) strain by microarray analysis.

Seq_ID	Gene	Description	$\Delta fliC$	$\Delta fliD$	$\Delta motABCD$	$\Delta fgt1$	$\Delta psyI$	$\Delta aefR$	$\Delta gacA$
PSPPH_1609	<i>orf5</i>	Rieske (2Fe–2S) domain-containing protein	0.00	0.00	0.03	0.01	0.01	0.01	0.04
PSPPH_1610	<i>orf4</i>	Hypothetical protein	0.00	0.00	0.01	0.00	0.00	0.00	0.04
PSPPH_1611	<i>orf3</i>	Dihydrodipicolinate synthetase family protein	0.00	0.00	0.01	0.00	0.00	0.01	0.02
PSPPH_1612	<i>orf2</i>	Hypothetical protein	0.00	0.00	0.01	0.00	0.00	0.01	0.02
PSPPH_1613	<i>orf1</i>	Hypothetical protein	0.00	0.00	0.01	0.00	0.00	0.01	0.03
PSPPH_1614	<i>psyI</i>	Autoinducer synthesis protein Psyl (AhlI)	0.02	0.02	0.11	0.08	0.03	0.06	0.16
PSPPH_1615	<i>psyR</i>	Autoinducer transcriptional regulator PsyR (AhlR)	0.06	0.15	0.26	0.45	0.32	0.38	0.26
PSPPH_1617	<i>marR</i>	MarR family transcriptional regulator	0.38	0.12	0.29	0.32	0.30	0.21	0.37

Numbers indicate fold induction of each transcript accumulation relative to the respective controls (WT strain).

Fig. 1 Schematic organization of *marR*, *orf5* and surrounding genes in *Pseudomonas syringae* pv. *phaseolicola* 1448A (Pph1448A) and construction of $\Delta marR$ and $\Delta orf5$ mutants in the corresponding region of *P. syringae* pv. *tabaci* 6605 (Pta6605). Seq_IDs for Pph1448A are shown by the respective arrows. Polymerase chain reaction (PCR) was carried out to isolate each gene containing a full open reading frame and additional upstream and downstream regions. DNA fragments were obtained using primers (marR-5 and marR-3, and orf5-5 and orf5-3, respectively), indicated by arrowheads; the internal region was then excised.

Cloning of genes for MarR and Rieske (2Fe–2S) cluster-containing protein and similarity to their related proteins

The corresponding regions of *marR* and *orf5* were cloned by polymerase chain reaction (PCR) from the genomic DNA of Pta6605, as shown in Fig. 1. The deduced MarR and Orf5 proteins have 150 and 78 amino acid residues, with molecular masses of 17.2 and 8.9 kDa, respectively. MarR showed 100%, 92.7% and 92.7% identity with that of *P. syringae* pv. *phaseolicola* 1448A (Pph1448A), *P. syringae* pv. *syringae* B728a (PsyB728a) and *P. syringae* pv. *tomato* DC3000 (PtoDC3000), respectively (Fig. S1, see Supporting Information). The crystal structure of MarR has been reported as a dimer, with each subunit containing a common triangular shape with a winged-helix DNA-binding motif, although members of this family have few sequence similarities (Wilkinson and Grove, 2006). According to the secondary structure predicted by the well-known prediction server PHD (<https://npsa-pbil.ibcp.fr>) (Rost and Sander, 1993), MarR of Pta6605 is rich in helices. The $\Delta marR$ mutant generated as shown in Fig. 1 lost most of its DNA-binding motif, as shown in Fig. S1A. Although some plant-associated bacteria, such as *P. fluorescens* SBW25 and *P. protegens* Pf-5, possess the corresponding orthologue of MarR, it is not common in pseudomonads and is absent in *P. aeruginosa*. Orf5 showed 100%, 96.1% and 87.2% identity with Pph1448A, PsyB728a and PtoDC3000, respectively. Orf5 possesses the Rieske motif, which is involved in electron transfer. Corresponding amino acids (C28, C49, H30 and H52) are conserved in Orf5 of Pta6605 (Fig. S1B), but the $\Delta orf5$ mutant generated in this study lost all of these as a result of a frame shift. Orf5 is specific to *P. syringae*, and there is no corresponding orthologue in other species.

Effect of the exogenous application of *N*-hexanoyl-DL-homoserine lactones in the *psyl* mutant

To investigate the effect of the exogenous application of *N*-hexanoyl-DL-homoserine lactones on *marR* and *orf5* expression in the *psyl* mutant, microarray analyses and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) were carried out (Fig. 2; Table S2 and Fig. S2, see Supporting Informa-

tion). The results clearly demonstrated that the expression of both *marR* and *orf5* genes in the *psyl* mutant fully recovered to the same level as the WT strain after treatment with 1 mM *N*-hexanoyl-DL-homoserine lactone, suggesting that the expression of *marR* and *orf5* genes is under the regulation of AHL-mediated QS.

AHL production in $\Delta marR$ and $\Delta orf5$ mutant strains

We examined the production of AHL in the $\Delta marR$ and $\Delta orf5$ mutant strains using *Chromobacterium violaceum* CV026, a biosensor strain. We observed that there was no significant difference in the amount of AHL produced in each strain (Fig. S3, see Supporting Information).

Survival assays to oxidative or nitrosative stress

It has been reported that MarR family members alter the cell surface to protect it from toxic compounds produced by the host or environmental stimuli (Ellison and Miller, 2006). To investigate the effect of *marR* or *orf5* mutation in response to oxidative and nitrosative stress, tolerance assays were performed. As shown in Fig. 3, stationary phase cells of the $\Delta marR$ and $\Delta orf5$ mutant strains showed significantly lower tolerance to 0.3% H_2O_2 than the cells of the WT strain. Similarly, when each strain was exposed to 550 and 750 μM *S*-nitrosoglutathione as an NO donor, both mutant strains showed greater sensitivity to NO stress than the WT strain. These phenotypes of the $\Delta marR$ and $\Delta orf5$ mutant strains were almost restored in each complemented strain (Fig. 3). These results clearly demonstrate that *marR* and *orf5* are required for tolerance against oxidative and nitrosative stress.

Motility experiments and Western blotting of flagellin protein

A MarR family transcriptional regulator, SlyA, in *D. dadantii* 3937 has been reported to be involved in the control of swimming motility (Zou *et al.*, 2012). Therefore, flagellum-mediated surface swimming and swarming motility assays of each mutant and WT strain of Pta6605 were performed on 0.25% agar minimal medium with mannitol and fructose (MMMF) and 0.45% agar

Fig. 2 Effect of exogenous application of *N*-hexanoyl-DL-homoserine lactone (acyl homoserine lactone, AHL) on *marR* and *orf5* expression in wild-type (WT) and $\Delta psyI$ mutant. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA prepared from each strain. The data presented are the representative results obtained from three replicates. EtOH, ethanol.

swarming (SWM) plates, respectively. As shown in Fig. 4A, B, the motilities of the mutant strains were strongly reduced, whereas those of the complemented strains were almost completely restored. The addition of membrane-protecting agents, such as sorbitol at 10 mM final concentration and glycerol at 2.5% final concentration, did not rescue surface swarming motility (data not shown). To compare the amount of flagellin protein in whole-cell lysates from each strain, immunoblotting with an anti-flagellin antibody was performed (Fig. 4C). Quantitative analysis demonstrated that a larger amount of flagellin protein was detected in the WT and complemented strains relative to that in both $\Delta marR$ and $\Delta orf5$ mutants. However, the result of microarray analysis showed no significant difference in the expression of flagellar biogenesis-related genes, including *fliC*, between the WT and each mutant strain (Table S3, see Supporting Information). Therefore, we compared the amount of flagellin protein in the supernatant of each bacterial culture (Fig. 4D). Larger amounts of flagellin protein were detected in both mutant strains, suggesting that flagella are released from the cell surface of both mutants.

Detection of live or dead cells

Because the results shown in Figs 3 and 4 suggest that the cell membranes of $\Delta marR$ and $\Delta orf5$ mutants may be damaged, live and dead cells in each strain were quantitatively assessed using

the LIVE/DEAD BacLight bacterial viability assay kit, as described in Experimental procedures. After stationary incubation in King's B (KB) liquid medium overnight, plentiful green fluorescent bacteria were observed in the WT and complemented strains, whereas the majority of $\Delta marR$ and $\Delta orf5$ mutant bacteria were fluorescent red (Fig. 5A). The quantitative analysis demonstrated that more than 70%, approximately 40% and 20% of cells were dead in each mutant, WT and complemented strain, respectively, in this condition (Fig. 5B). Figure 5C demonstrates the cell surface structure of WT, $\Delta marR$ and $\Delta orf5$ mutant strains by TEM. The photographs show that the cell surface in each mutant is severely damaged with multiple ruptures. These results suggest that disruption of *marR* and *orf5* may affect the expression of essential components of the cell membrane and lead to cell death.

Growth experiment

Because the percentage of dead cells is higher in $\Delta marR$ and $\Delta orf5$ mutants than in the WT strain (Fig. 5B), we monitored the growth of $\Delta marR$ and $\Delta orf5$ mutant strains in KB liquid medium by measuring the optical density (OD) at 660 nm for 24 h. The doubling times of WT, $\Delta marR$ and $\Delta orf5$ strains were 97.15 ± 0.21 , 101.5 ± 1.90 and 97.4 ± 3.88 min, respectively, during the early exponential phase, with no significant difference. However, the final concentration after 24 h of incubation of the WT reached $OD_{660} = 3.86$, whereas the values of the $\Delta marR$ and $\Delta orf5$ mutants were 3.24 and 3.49, respectively. These results suggest that both mutant strains exhibit growth defects at the stationary phase (Fig. S4, see Supporting Information).

Detection of extracellular DNA and protein

Because defective *marR* and *orf5* expression damaged the cell membrane structure, as shown in Figs 3–5, we determined the levels of extracellular DNA and proteins released from each bacterium into the culture supernatant during overnight planktonic incubation. Absorbances at 260 and 280 nm of the cell-free supernatant were assessed as the concentrations of extracellular DNA and protein, respectively. The results demonstrated that the concentrations of both extracellular DNA and protein in the supernatants of $\Delta marR$ and $\Delta orf5$ mutants were higher than in the other strains, suggesting that large amounts of DNA and proteins are released from the membrane-damaged and/or dead cells of the mutants into the extracellular medium (Fig. 6).

Virulence of $\Delta marR$ and $\Delta orf5$ mutants on host tobacco leaves

To assess the significance of *marR* and *orf5* in the virulence of *Pta6605*, an inoculation test of host tobacco leaves with the WT, each mutant and complemented strain was performed. As shown in

Fig. 3 Survival assay of oxidative (A) and nitrosative (B) stress. Stationary phase cells were exposed to 0.3% H₂O₂ and 550 or 750 μM S-nitrosoglutathione for 30 min at 27 °C, and the viable cell number was determined by plating on KB plates. The relative number of colonies in the wild-type (WT) was set to unity. The experiment was repeated three times independently. Asterisk indicates significant difference from the WT (**P* < 0.05).

Fig. 7A, both mutants caused mild disease symptoms relative to the WT strain, and both complemented strains had recovered the ability to cause severe disease symptoms. The bacterial populations of the WT and complemented strains in host tobacco leaves were around 10^6 and 4×10^6 colony-forming units (cfu)/cm² at 2 and 6 days after inoculation, respectively, whereas those of each mutant strain were significantly lower than those of the WT and complemented strains (Fig. 7B). Because there is no difference in the growth rate of each strain in KB medium during the early exponential phase (Fig. S4), we conclude that the $\Delta marR$ and $\Delta orf5$ mutant strains in *Pta6605* show reduced virulence, and that both genes play an important role in virulence in host tobacco infection.

Gene expression profiles of $\Delta marR$ and $\Delta orf5$ mutants

To investigate the regulation mechanisms mediated by *marR* and *orf5* gene products, microarray analysis was performed under the conditions described in Experimental procedures. The result revealed that the gene expression profiles of $\Delta marR$ and $\Delta orf5$ mutants exhibited partial similarity, suggesting that both gene products are components of the same regulatory cascade (Table S3). A number of genes encoding O-antigen-, glutamine-, peptide- and taurine-ABC transporters, and other transporters, were repressed in both mutants (Table 2). In addition to the genes for these integral membrane components, a gene for capsular polysaccharide synthesis, *phoR* for a sensor histidine kinase for environmental inorganic phosphate and *nirD* for nitrite reductase were regulated in a *marR*- and *orf5*-dependent manner (Table 2). Although it has been reported that SlyA, a member of the MarR family in *D. dadantii* 3937, positively regulates the expression of *hrpA* and *hrpN*, two *hrp* genes located in the *hrpL* regulon, the expression of major T3SS genes was not affected in $\Delta marR$ and $\Delta orf5$ mutants. On the contrary, the expression of several genes encoding hypothetical proteins and the transposons *IsPsy18* were activated (Table S3).

Tolerance to antibiotics

Finally, the minimum inhibitory concentration (MIC) of the WT, $\Delta marR$ and $\Delta orf5$ mutants to antimicrobial reagents was exam-

ined using E test strips that contained predefined gradients of chloramphenicol, spectinomycin and cefotaxime. MIC was determined by detecting the growth inhibition of each strain on KB agar plates after 24 h of incubation, according to the manufacturer's protocol (BioMerieux, Craponne, France). However, no significant difference in the tolerance to these antibiotics was observed for the WT and each mutant (data not shown).

DISCUSSION

In this study, we investigated the mechanism of QS-regulated virulence in *Pta6605*. The expression of the genes corresponding to PSPPH_1609 to PSPPH_1615 and PSPPH_1617, including *marR* and *orf5*, was regulated by QS because the exogenous application of AHL restored the expression of these genes (Fig. 2, Table S2). It has been reported that *hpaR*, a member of *marR* in *Xanthomonas campestris* pv. *campestris* (Xcc), is an indispensable factor for virulence, and the expression of *hpaR* is positively controlled by HrpG and HrpX, regulators of *hrp* gene expression (Wei *et al.*, 2007). Thus, the expression of *hpaR* in Xcc and *marR* in *Pta6605* is differentially regulated.

The function of *marR* and the Rieske (2Fe–2S) cluster-containing protein gene (*orf5*) in virulence was characterized using each mutant strain in *Pta6605*. MarR belongs to a large antibiotic resistance transcriptional regulator family in bacteria and archaea (Perera and Grove, 2010). It has been reported that MarR regulates the expression of several genes in host organisms important in survival, resistance to oxygen-dependent antimicrobial systems and the production of membrane and periplasmic proteins for resistance to antimicrobial peptides (Ellison and Miller, 2006). Our results revealed that the $\Delta marR$ and $\Delta orf5$ mutants in *Pta6605* showed reduced survival in nitrosative or oxidative stress (Fig. 3), reduced surface swimming and swarming motilities (Fig. 4), decreased viability in liquid culture (Fig. 5A, B) and, consequently, lower virulence on host tobacco (Fig. 7). Electron microscopic observation revealed severely damaged cell surface structures in the $\Delta marR$ and $\Delta orf5$ mutants (Fig. 5C), suggesting that *marR* and *orf5* may regulate the expression of genes important for a robust membrane

Fig. 4 Surface motilities and flagellin production. Surface swimming motility (MMM with 0.25% agar) at 23 °C (A) and surface swarming motility (SWM with 0.45% agar) at 27 °C (B). Data shown in parentheses are the average of three independent replicates. Immunoblot analysis of flagellin protein in whole-cell lysates (C) and supernatant (D) from each strain using anti-flagellin antibody (top panels). The signal intensity was quantified using ImageJ 1.45 software. The bars represent standard deviations for three independent experiments. Asterisk indicates significant difference from the wild-type (WT) (* $P < 0.01$).

structure and survival in host plants. As the $\Delta marR$ and $\Delta orf5$ mutants produce a similar amount of AHL to the WT (Fig. S3), the phenotype of these mutants and QS-defective mutants, such as $\Delta psyl$ (Taguchi *et al.*, 2006a), is quite different. A MarR family transcriptional regulator, SlyA, in *Salmonella typhimurium* also regulates genes related to the production of membrane, periplasmic and secreted proteins, indicating that the major role

of MarR is to alter the cell surface for protection from harmful compounds, probably produced by host organisms or environmental stimuli (Ellison and Miller, 2006). Microarray analysis of $\Delta marR$ and $\Delta orf5$ mutant strains showed that the expression of many transporter-related genes was largely reduced in the mutants (Table 2), indicating the possible accumulation of harmful compounds in the cytoplasm.

Fig. 5 Detection of live or dead cells and transmission electron microscopy (TEM) observation of cell structure. (A) Bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. (B) The dead cells detected by this kit were counted under a fluorescence microscope. The ratio of dead cells among 1000 cells was calculated. The experiment was repeated three times independently. Asterisk indicates significant difference from the wild-type (WT) (* $P < 0.01$). (C) TEM image of cell membrane structure of each strain. Scale bar, 500 nm.

Among Rieske 2Fe–2S cluster-containing proteins, SoxR in *E. coli* has been reported to be a bifunctional protein that senses environmental conditions and responds to them as a transcriptional factor by the activation of a diverse set of genes (Shen and Fang, 2012). This protein contains a single 2Fe–2S cluster, which is required for the sensing of superoxide and nitric oxide. When the 2Fe–2S cluster of the SoxR transcription factor is oxidized by superoxide, SoxR induces the expression of SoxS, which is responsible for the expression of downstream genes for antioxidant defence. Furthermore, SoxR is directly activated by nitric oxide to form a dinitrosyl–iron–dithiol complex with 2Fe–2S clusters (Shen and Fang, 2012). Because the $\Delta orf5$ mutant in this study also reduced the tolerance to oxidative and nitrosative stress, the Rieske 2Fe–2S cluster in Orf5 may possess a similar role to SoxR as a sensor and transcriptional factor. Furthermore, the expression level of *nirD*, which encodes a small nitrite reductase [NAD(P)H] subunit, one of the enzymes that converts inorganic nitrate to nitrite and ammonium (Romeo *et al.*, 2012), was significantly reduced in the $\Delta marR$ and $\Delta orf5$ mutants relative to that in the WT

(Table 2); this suggests that *marR* and *orf5* are required for the expression of *nirD*, although the molecular mechanism of nitrate assimilation by *nirD* in *Pta6605* is not known.

To screen the target genes responsive to *N*-(3-oxohexanoyl)-L-homoserine lactone, random transposon mutagenesis with a *gusA* reporter construct was carried out in the QS-negative *expl* mutant of the plant pathogen *Erwinia carotovora* ssp. *carotovora* SCC3193, and two AHL-responsive genes that encode a regulator Hor and a novel plant ferredoxin-like protein with a 2Fe–2S conserved domain (FerE) were identified (Sjöblom *et al.*, 2008). Hor shows 75% identity to a MarR/SlyA transcriptional regulator of *Yersinia enterocolitica* ssp. *enterocolitica* 8081, whereas FerE shows a high similarity to cyanobacterial and plant ferredoxins, but lower homology to other bacterial proteins, suggesting acquisition through horizontal gene transfer. Both the *hor* and *ferE* mutants exhibit reduced virulence by the reduced production of plant cell wall-degrading enzymes and tolerance to oxidative stress. Although *marR* and *orf5* in *Pta6605* show no homology with these genes, a similar mechanism might control oxidative

Fig. 6 Measurement of extracellular DNA and protein contents. After overnight incubation of the wild-type (WT) and each strain at 27 °C, the absorbance of the cell-free supernatant at 260 and 280 nm was assessed as the amount of extracellular DNA and protein concentration, respectively. The experiment was repeated three times independently. Asterisk indicates significant difference from the WT (* $P < 0.05$).

stress tolerance and bacterial virulence under the regulation of QS. Sjöblom *et al.* (2008) have demonstrated that the two ExpR QS regulators (ExpR1 and ExpR2) negatively regulate the expression of *hor* and *ferE* via the global negative regulator RsmA, an RNA-binding protein. Further investigation of a global virulence regulatory mechanism mediated by QS with *marR* and *orf5*, and the potential contribution of RsmA in *Pta6605*, is required in future work.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth condition

All bacterial strains used in this study are listed in Table 3. The *Pta6605* strains were maintained as described by Taguchi *et al.* (2006b). The *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 a final concentration of 50 μg/mL kanamycin (McClellan *et al.*, 1997).

Plant materials, inoculation procedure and bacterial growth

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 25 °C with a 12-h photoperiod. The dip inoculation of host tobacco leaves was performed as described previously (Taguchi and Ichinose, 2011). The inoculated leaves were incubated under conditions of 100% humidity for 1 day and 85% humidity for 10 subsequent days at 23 °C with an 18-h photoperiod. For the determination of bacterial multiplication, five leaf discs (diameter, 8 mm) were punched from tobacco leaves and soaked for 20 min in a suspension of bacteria in 10 mM MgSO₄ with 0.02% silwet at

a density of 2×10^8 cfu/mL. The inoculated leaf discs were incubated on Whatman 3MM paper (GE Healthcare, Little Chalfont, UK) with a distilled water supply. The bacterial population from inoculated leaves was determined as described by Taguchi and Ichinose (2011).

Construction of mutants and their complemented strains

To generate *marR*- and *orf5*-defective mutants, the genetic region including each gene was amplified using a set of PCR primers (*marR*-5, 5'-ATGAACAGCGCAAATGGCCA-3'; *marR*-3, 5'-ATGACCTTGCTGTACCACG-3'; *orf5*-5, 5'-TTGCCAGTTCAGCCATCCC-3'; *orf5*-3, 5'-TGGTGATCTCGCTGGTCTGC-3'), which were designed on the basis of the registered sequence of each gene of *Pta6605* (Fig. 1), and inserted into a pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA). The recombinant plasmids obtained were digested with *Sall* and *NruI* to delete the internal 288 bp for the generation of the *ΔmarR* mutant plasmid, and *MfeI* and *kpnI* to delete the internal 113 bp for the generation of the *Δorf5* mutant plasmid. After digestion with the respective restriction enzymes, purified DNA was treated with DNA polymerase (*Pyrobest* DNA polymerase, Takara, Kyoto, Japan) to generate blunt ends and then self-ligated by a T4 DNA ligase (Ligation-Convenience kit, Nippon Gene, Tokyo, Japan). Each mutated genetic region was introduced into a pK18*mobsacB* plasmid via an *EcoRI* site (Schäfer *et al.*, 1994). Deletion mutant strains were obtained by conjugation and homologous recombination according to the methods reported previously (Taguchi *et al.*, 2006b). To confirm whether the altered phenotype originates from a corresponding mutation, each complemented strain was generated. For the *ΔmarR* mutant, pCR-Blunt II-Topo containing the PCR product described above was used for complementation because the predicted promoter and entire open reading frame were included in this region. For the *Δorf5* mutant, the genetic region from *orf1* to *orf5* and predicted promoter were isolated by pCR-Blunt II-TOPO vector using a set of PCR primers (*orf5*-F1, 5'-AACTCAAACCCGCTCGACAT-3'; *orf5*-R1, 5'-CTACCTCAGTCGCCGAACCT-3') and cloned into the conventional plasmid vector. The resulting plasmids were ligated with pBSL118, a transposon vector (Alexeyev *et al.*, 1995), at the *EcoRI* site to generate pBSL-*marR* and pBSL-*orf5*, respectively, and were introduced into the respective mutant by conjugation using the *E. coli* S17-1 λpir strain to generate the complemented strains *marR*-C and *orf5*-C, respectively. The DNA sequences of the mutated and complemented regions in each bacterium were confirmed with a DNA sequencer (ABI PRISM 3100, Applied Biosystems, Tokyo, Japan) using a BIG Dye terminator cycle sequencing kit.

Motility assay

Motility experiments were performed as described previously (Taguchi *et al.*, 2006b, 2010). The surface swimming motility was observed on 0.25% agar MMMF (50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂ and 1.7 mM NaCl, pH 5.7, supplemented with 10 mM each of mannitol and fructose) plates after 24 and 72 h at 23 °C, and the surface swarming motility was assessed on 0.45% agar SWM (0.5% peptone and 0.3% yeast extract) plates after 24 h of incubation at 27 °C. For the quantification of motilities, the diameters of the swimming and swarming areas were measured at 72 and 24 h, respectively, after inoculation.

Fig. 7 Inoculation of host tobacco leaves by the dip method. (A) Tobacco leaves were dip inoculated with each bacterial suspension [2×10^8 colony-forming units (cfu)/mL in 10 mM $MgSO_4$] for 20 min at 23 °C. Photographs taken 11 days after inoculation show the representative results obtained from three independent experiments. (B) Bacterial populations were calculated at 0, 2 and 6 days post-inoculation. The bars represent standard deviations for three independent experiments. Asterisk indicates significant difference from the wild-type (WT) (* $P < 0.01$).

Table 2 Significantly affected gene expression in $\Delta orf5$ and $\Delta marR$ mutant strains.

Seq_ID	Gene	Description	$\Delta orf5$	$\Delta marR$
PSPPH_4684		O-antigen ABC transporter, permease protein, putative	0.15	0.31
PSPPH_2462		Glutamine ABC transporter, permease protein	0.08	0.09
PSPPH_2695		Peptide ABC transporter, permease protein	0.06	0.19
PSPPH_4910		Taurine ABC transporter, permease protein	0.05	0.53
PSPPH_4063		Amino acid transporter LysE	0.06	0.05
PSPPH_3045		AcrB/AcrD/AcrF family transporter	0.08	0.16
PSPPH_2379		Multidrug efflux transporter	0.15	0.56
PSPPH_3150		Capsular polysaccharide biosynthesis protein	0.11	0.10
PSPPH_5115	<i>phoR</i>	Sensory box sensor histidine kinase PhoR	0.07	0.07
PSPPH_3012	<i>nirD</i>	Nitrite reductase [NAD(P)H], small subunit	0.25	0.31

Tolerance to antibiotics

The MIC of antimicrobial reagents was determined with Etest strips containing a predefined gradient of chloramphenicol, spectinomycin and cefotaxime by detecting the growth inhibition of each strain on KB agar

plates after 24 h of incubation according to the manufacturer's protocol (BioMerieux).

RNA preparation, microarray analysis and semi-quantitative RT-PCR

Each bacterial strain was grown in LB medium with 10 mM $MgCl_2$ at 27 °C to an OD_{600} of 0.3. The cells collected by centrifugation were further incubated in MMMF medium for an additional 1 h. Total RNA was extracted using a High Pure RNA Isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Total RNA (20 μ g) was used for microarray analysis employing a Roche NimbleGen microarray system, as described previously (Taguchi and Ichinose, 2011).

To examine the effect of the exogenous application of *N*-hexanoyl-DL-homoserine lactone (Sigma-Aldrich, St. Louis, MO, USA) on *marR* and *orf5* expression, WT and *psyl* mutant strains were grown in LB medium with 10 mM $MgCl_2$ at 27 °C overnight. The cells collected after centrifugation were adjusted to an OD_{600} of 0.1 by MMMF medium with or without 1 mM of *N*-hexanoyl-DL-homoserine lactone, and the cultures were further incubated for an additional 24 h at 27 °C. As a control treatment, the same amount of ethanol was applied. Semi-quantitative RT-PCR analysis was conducted using 2 μ g of total RNA. The specific primer sets (*marR*-1, 5'-GGATACGTGGTTCAGATTCG-3'; *marR*-2, 5'-ACATCTGTTTGCAGTGTG-3'; *orf5*-1, 5'-TTGAACGAGCCTGCTGAGTT-3'; *orf5*-2, 5'-CTACCTCAGTCGCGAACCT-3') were designed according to the registered sequences of

Table 3 Bacterial strains and plasmids.

Bacterial strain/plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i> DH5 α S17-1	F ⁻ λ - ϕ 80dLacZ Δ M15 Δ (lacZYA-argF)U169 recA1endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA relA1 thi pro hsdR hsdM ⁺ recA(chr::RP4-2-Tc::Mu-Km::Tn7)	Takara, Kyoto, Japan Schäfer <i>et al.</i> (1994)
<i>Chromobacterium violaceum</i> CV026	Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC31532, AHL biosensor	McClellan <i>et al.</i> (1997)
<i>Pseudomonas syringae</i> pv. <i>tabaci</i> Isolate 6605 6605-marR 6605-orf5 6605-marR (pBSL118 marR) 6605-orf5 (pBSL118 orf5)	Wild-type isolated from tobacco, Nal ^r Isolate 6605 vfr, Nal ^r Isolate 6605 cyaA, Nal ^r Isolate 6605 marR, Nal ^r (pBSL-marR) Isolate 6605 orf5, Nal ^r (pBSL-orf5)	Shimizu <i>et al.</i> (2003) This study This study This study This study
Plasmids pCR-BluntII-TOPO pCR-BluntII-TOPO-marR pCR-BluntII-TOPO-orf5 pK18mobSacB pK18-marR pK18-orf5 pBSL118 pBSL-marR pBSL-orf5	Cloning vector, Kmr marR-containing 453-bp DNA fragment in pCR-BluntII-TOPO orf5-containing 237-bp DNA fragment in pCR-BluntII-TOPO Small mobilizable vector, Km ^r , sucrose sensitive (sacB) Defective marR-containing DNA fragment in pK18mobSacB Defective orf5-containing DNA fragment in pK18mobSacB Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp ^r , Km ^r marR-containing 2524-bp DNA fragment in pBSL118 Orf5-containing 3809-bp DNA fragment in pBSL118	Invitrogen, Carlsbad, CA This study This study Schäfer <i>et al.</i> (1994) This study This study Alexeyev <i>et al.</i> (1995) This study This study

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

Pta6605. PCR was performed as follows: one denaturation cycle of 2 min at 95 °C; 27 cycles of 30 s at 95 °C, 30 s at 60 °C and 50 s at 72 °C. Ten microlitres of the PCR product were loaded onto 1.5% agarose gel. RT-PCR was also carried out without Avian Myeloblastosis Virus (AMV) RTase as a negative control.

TEM observation of membrane structure

Samples for TEM observation were prepared from overnight cultured cells. Each cell type was collected by centrifugation and fixed on ice for 2 h with a mixture of 2.5% glutaraldehyde, 1% OsO₄ and 4.5% sucrose in 0.1 M cacodylate buffer (pH 7.0), and then treated with 0.2% tannic acid solution for 10 min. The fixed samples were washed with distilled water, followed by embedding in 3% agar. Small pieces of agar block were dehydrated through a graded ethanol series before being embedded in Quetol 651 resin mixture (NEM, Tokyo, Japan). Ultrathin sections (70 nm) were cut from the resin blocks and mounted onto Cu grids for observation using a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) operated at 80 kV (Suzuki *et al.*, 2012).

Detection of AHLs

Extraction of AHLs and their detection using *C. violaceum* CV026 were performed as described previously (Taguchi *et al.*, 2010).

Quantification of extracellular DNA and protein

Each bacterial strain was grown in LB medium with 10 mM MgCl₂ at 27 °C for 24 h, and the bacterial suspension was adjusted to an OD₆₀₀ of 1.0. After centrifugation, supernatants of each culture were filtered through a 0.45- μ m filter. To quantify the amount of extracellular DNA and protein, the absorbances of the culture supernatant were measured at 260 and 280 nm, respectively.

Detection of living and dead cells

To compare viability, WT and each mutant strain were grown in KB liquid culture at 27 °C for 18 h. After centrifugation, the cell pellet was resuspended in 0.9% NaCl. This washing step was repeated and the suspension was adjusted to an OD₆₀₀ of 0.2. Cells were stained with SYTO 9 and propidium iodide using a LIVE/DEAD BacLight™ bacterial viability kit (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The live and dead cells were detected by green and red fluorescence, respectively, under a fluorescence microscope (OLYMPUS IX70; Tokyo, Japan). The ratio of dead cells among 1000 cells was calculated.

Immunoblot analysis

Immunoblot analysis was carried out as described previously (Taguchi *et al.*, 2006b). For detection of the flagellin protein in cell fractions, an overnight culture of each bacterium in LB with 10 mM MgCl₂ at 27 °C was further incubated in MMMF at 23 °C for 24 h, and the supernatant and cells were harvested by centrifugation. The whole-cell lysate was prepared by dissolving the cell pellet in sample buffer, and total protein from the culture supernatant was precipitated by the addition of one-fifth volume of 10% trichloroacetic acid and dissolved in sample buffer for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation of total proteins on a 12.5% SDS-PAGE gel, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK), and immunoblot analysis was carried out using a rabbit polyclonal anti-flagellin-specific antibody with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA, USA) and an alkaline phosphatase-based chemiluminescent detection system (CDP-Star Reagent, NEB, Beverly, MA, USA), according to the manufacturer's instructions. The signal intensity from the gel image by immunoblot analysis was quantified using ImageJ 1.45 software (National Institutes of Health, Bethesda, MD, USA).

Survival assays under oxidative or nitrosative stress

To examine tolerance to oxidative or nitrosative stress, each bacterial strain was grown overnight in liquid KB medium at 27 °C. The cells were harvested and adjusted to an OD₆₀₀ of 0.5 in 10 mM MgSO₄. Each cell suspension was exposed to a final concentration of 0.3% H₂O₂ for 30 min at room temperature, and viable cell numbers were counted by plating on KB plates. Similarly, tolerance to NO was compared in the presence of 750 or 550 µM S-nitrosoglutathione. All experiments were carried out in triplicate.

Statistical analysis

The results of bacterial populations in host tobacco leaves, motilities, flagellin proteins detected by Western blot, bacterial viability assay, quantification of extracellular DNA and protein, and survival assay are expressed as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were performed using KaleidaGraph version 4.02 (Synergy Software, Reading, PA, USA) for comparison between the quantitative measurements of the WT, mutants and complemented strains. *P* < 0.05 was considered to be statistically significant.

Nucleotide sequence accession numbers

The nucleotide sequences of the *marR* and *orf5* genes were deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases by Dr D. Studholme (University of Exeter, Exeter, UK) under the accession numbers AJX101000167.1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 (A) Multiple sequence alignment of deduced amino acid sequences of MarR from Pta_MarR (*Pseudomonas syringae* pv. *tabaci* 6605, Pta6605), PSPPH_1617 (*P. syringae* pv. *phaseolicola* 1448A, Pph1448A), PSPTO_3861 (*P. syringae* pv. *tomato* DC3000, PtoDC3000), Psyr_1624 (*P. syringae* pv. *syringae* B728a, PsyB728a), PFLU_3919 (*P. fluorescens* SBW25), PFL_3976 (*P. protegens* Pf-5) and L487_14415 (*P. putida* H8234). Identical amino acids are indicated by asterisks below the sequence. Predicted regions for the α -helix of Pta_MarR are shown with + above the sequence. Deleted region is indicated by shaded letters. (B) Multiple sequence alignment of deduced amino acid sequences of Orf5 from Pta_Orf5 (Pta6605), PSPPH_1609 (Pph1448A), PSPTO_3869 (PtoDC3000) and Psyr_1616 (PsyB728a). The two conserved cysteine residues (C) and two histidine residues (H) in the Rieske motif are shown above the

sequences. The region disrupted by deletion and frame shift is indicated by shaded letters.

Fig. S2 Effect of exogenous application of low concentration of *N*-hexanoyl-DL-homoserine lactone (acyl homoserine lactone, AHL) on *marR* and *orf5* expression in the Δ psyl mutant. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA prepared from each strain. The data presented are the representative results obtained from three replicates.

Fig. S3 Detection of acyl homoserine lactones (AHLs) using *Chromobacterium violaceum* CV026 as biosensor strain. Ten-microlitre aliquots of 500-fold-concentrated extracts in ethyl acetate from each strain were developed by C_{18} reverse-phase thin layer chromatography (TLC).

Fig. S4 Growth curve of wild-type (WT), Δ marR and Δ orf5 mutants in KB liquid medium. The growth of each strain was monitored by measuring the optical density at 660 nm during 24 h of incubation using a compact rocking incubator (ADVANTEC, TVS062CA). The data presented are the representative results from two replicates.

Table S1 Gene expression profiles of AHL production-defective mutants compared with those of WT strain by microarray analysis.

Table S2 Gene expression profiles of Δ psyl and Δ psyl with 1 mM AHL compared with those of WT strain by microarray analysis.

Table S3 Gene expression profiles of Δ marR and Δ orf5 mutant strains compared with those of WT strain by microarray analysis.