Virulence factor regulator (Vfr) controls virulence-associated phenotypes in *Pseudomonas syringae* pv. *tabaci* 6605 by a quorum sensing-independent mechanism

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SUMMARY

Virulence factor regulator (Vfr) is a member of the cyclic 3',5'-adenosine monophosphate (cAMP) receptor proteins that regulate the expression of many important virulence genes in *Pseudomonas aeruginosa*. The role of Vfr in pathogenicity has not been elucidated fully in phytopathogenic bacteria. To investigate the function of Vfr in *Pseudomonas syringae* pv. *tabaci* 6605, the *vfr* gene was disrupted. The virulence of the *vfr* mutant towards host tobacco plants was attenuated significantly, and the intracellular cAMP level was decreased. The *vfr* mutant reduced the expression of flagella-, pili- and type III secretion system-related genes and the defence response in nonhost *Arabidopsis* leaves. Furthermore, the expression levels of achromobactin-related genes and the iron uptake ability were decreased, suggesting that Vfr regulates positively these virulence-related genes. In contrast, the *vfr* mutant showed higher tolerance to antimicrobial compounds as a result of the enhanced expression of the resistance–nodulation–division family members, the *mexA*, *mexB* and *oprM* genes. We further demonstrated that the mutant strains of *vfr* and *cyaA*, an adenylate cyclase gene responsible for cAMP synthesis, showed a similar phenotype, suggesting that Vfr regulates virulence factors in a cAMP-dependent manner. Because there was no significant difference in the production of acylhomoserine lactone (AHL) quorum sensing molecules in the wild-type, *vfr* and *cyaA* mutant strains, Vfr might control important virulence factors by an AHL-independent mechanism in an early stage of infection by this bacterium.

INTRODUCTION

It has been reported that intracellular cyclic 3',5'-adenosine monophosphate (cAMP) levels in the animal pathogen *Pseudomonas aeruginosa* modulate the expression of type III secretion system (T3SS) genes, and that a host cell contact signal mediates the expression of T3SS genes through the cAMP pathway (Wolfgang *et al.*, 2003; Yahr and Wolfgang, 2006). Many virulence factors,

including type IV pili (T4P), the type II secretion system, T3SS, quorum sensing and flagellar biogenesis, have been reported to be regulated by cAMP, and cAMP may control gene expression via the transcription factor Vfr (virulence factor regulator), which belongs to the cAMP receptor protein (Crp) family (Serate *et al.*, 2011; Whitchurch *et al.*, 2005). A recent article has reported that cAMP is required for DNA-binding activity and for the Vfr-dependent regulation of specific virulence genes (Fuchs *et al.*, 2010). There are relationships between quorum sensing and Vfr-mediated regulation. For example, Vfr in *P. aeruginosa* regulates directly *rhlR* transcription in the *rhl* quorum sensing system by binding to the Vfr-binding sites in the *rhlR* promoter (Croda-García *et al.*, 2011). However, the *Pseudomonas* quinolone signal [2-heptyl-3-hydroxy-4(1H)-quinolone: PQS] is regulated by the LasI and RhlI quorum sensing systems in *P. aeruginosa* (Diggle *et al.*, 2003), and Vfr may regulate all quorum sensing systems in *P. aeruginosa* (Whitchurch *et al.*, 2005). In *P. aeruginosa*, the Chp chemotaxis-like chemosensory system, which has been reported to regulate T4P production and twitching motility, plays a global regulatory role in virulence by modulating the intracellular cAMP level and the expression of Vfr-dependent virulence-related genes (Fulcher *et al.*, 2010).

The role of Vfr in pathogenicity has not been elucidated fully in phytopathogenic bacteria. In *Pectobacterium chrysanthemi*, pectinases that cleave pectin, a major component of the plant cell wall, are known to be important virulence factors. Previous studies have demonstrated that pectinase production is subjected to catabolite repression and that Crp is required for the expression of pectinase genes in this bacterium (Reverchon *et al.*, 1997). Clp, a Crp orthologue in *Xanthomonas campestris* pv. *campestris* (Xcc), has high homology with Crp in *Escherichia coli* and Vfr in *P. aeruginosa*, which contains a conserved sequence of the cyclic nucleotide monophosphate (cNMP)-binding domain (He *et al.*, 2007). It has been reported that Clp binds to the target DNA promoter, and that this DNA-binding ability is reduced by cyclic 3',5'-diguanosine monophosphate (c-di-GMP), indicating that Clp regulates negatively the expression of virulence genes by c-di-GMP in a dose-dependent manner (Chin *et al.*, 2010).

Previously, we have investigated the pathogenicity factors in *Pseudomonas syringae* pv. *tabaci* 6605 (Pta6605), a Gram-negative bacterium responsible for wildfire disease in host tobacco plants. This phytopathogenic bacterium requires many

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pathogenicity factors to establish infection. For example, flagella- and T4P-mediated motilities, T3SS, quorum sensing and Gac two-component systems are required for the virulence of this pathogen on host tobacco (Ichinose *et al.*, 2003; Marutani *et al.*, 2005, 2008; Taguchi and Ichinose, 2011; Taguchi *et al.*, 2006a). However, the mechanism that regulates the expression of virulence-related genes has not yet been elucidated. Recently, we have found that the expression of the *vfr* gene is reduced significantly in T4P-related mutants in *Pta6605* (Taguchi and Ichinose, 2011). Thus, the expression and function of T4P and Vfr may interact with each other to regulate virulence. However, little is known about Vfr in *Pta6605*.

In this study, to clarify the role of Vfr in virulence, we generated the mutant strain of *vfr* and its complemented strain in *Pta6605*. Furthermore, we also generated the mutant strain of *cyaA*, an adenylate cyclase gene responsible for cAMP synthesis, and its complemented strain to compare the phenotypes of *vfr* and *cyaA* mutant strains, and to analyse the involvement of cAMP in virulence. The phenotype of the *vfr* and *cyaA* mutants resembled that of T4P-defective mutants (Taguchi and Ichinose, 2011) as a result of the reduced surface motility, callose deposition in the nonhost plant and expression of T3SS genes, as well as the enhanced expression of the resistance–nodulation–division (RND) family members *mexA*, *mexB* and *oprM* (*mexAB/oprM*), which together are known as a major multidrug efflux system in *P. syringae* (Stoitsova *et al.*, 2008). On the basis of the results obtained in this study and our previous investigation, we discuss the regulatory network for the expression of virulence factors mediated by Vfr with cAMP and quorum sensing in the control of T4P and flagella biogenesis, respectively.

RESULTS

Cloning of *vfr* gene and similarity of Vfr and related proteins

Because many of the virulence factors have been shown to be regulated by Vfr in animal and plant pathogenic bacteria, we cloned *vfr* of *Pta6605* by polymerase chain reaction (PCR) from genomic DNA as described in Experimental procedures. The deduced Vfr protein has 214 amino acid residues with a molecular mass of 24.0 kDa, and shows 99%, 96% and 92% identity with that of *P. syringae* pv. *phaseolicola* 1448A (*Pph1448A*), *P. syringae* pv. *syringae* B728a (*PsyB728a*) and *P. syringae* pv. *tomato* DC3000 (*PtoDC3000*), respectively (data not shown). The amino acid residues G71, E72, R82, S83, T127 and S128 of Crp in *E. coli* are indispensable sites in cAMP binding (He *et al.*, 2007; Lee *et al.*, 1994). All of these amino acids, except for S128, are conserved in the Vfr proteins in *Pta6605* and *P. aeruginosa* (Fig. 1B, West *et al.*, 1994). The Vfr in *Pta6605* shows 88%, 71% and 46% amino acid identity with the Vfr in *P. aeruginosa* PAO1 (West *et al.*, 1994), Crp

in *E. coli* (Lee *et al.*, 1994) and Clp in *Xcc* (He *et al.*, 2007), respectively. The helix–turn–helix motif (R174 to K193 of Vfr in *Pta6605*), implicated in DNA binding (He *et al.*, 2007), is also conserved in *Pta6605*. The *vfr* mutant was constructed as described in Experimental procedures, which lost the predicted cAMP-binding sites (R86, S87, T131 and T132 of Vfr in *Pta6605*) and the DNA-binding site as a result of the frame shift.

Virulence of *vfr* mutant on host tobacco leaves

To investigate whether Vfr in *Pta6605* is involved in the expression of virulence factors, as reported for *P. aeruginosa* (Whitchurch *et al.*, 2005), an inoculation test of the wild-type (WT), *vfr* mutant and its complemented strain on host tobacco leaves was performed. As illustrated in Fig. 2A, the *vfr* mutant showed attenuated virulence compared with the WT strain, and the complemented strain recovered the ability to cause disease symptoms at 11 days post-inoculation (dpi). The bacterial population of the *vfr* mutant 3 and 6 dpi on host tobacco leaves was lower than that of the WT and complemented strains (Fig. 2B). Because the *vfr* mutant and complemented strain were able to grow at the same rate as the WT strain in Luria–Bertani (LB) medium with 10 mM MgCl₂ (data not shown), we concluded that *vfr* in *Pta6605* contributes to virulence in its host tobacco plant.

Motility experiments and Western blotting of flagellin protein

It has been reported that Vfr is a negative regulator of the major flagellar transcriptional regulator *fleQ* in *P. aeruginosa* (Dasgupta *et al.*, 2002). In contrast, Clp in *Xcc* has been reported to positively regulate the expression of the flagellin gene *fliC* (Lee *et al.*, 2003). The *vfr* mutant in *Pta6605* retained swimming motility in KB liquid medium (2% proteose peptone No.3, 8.6 mM K₂HPO₄, 1.6 mM MgSO₄, 1% glycerol) at the same level as the WT (data not shown). Therefore, the swimming motility of the *vfr* mutant on a 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 mannitol and fructose] plate and the swarming motility on a 0.45% agar SWM (0.5% peptone, 0.3% yeast extract) plate were compared with those of the WT in *Pta6605*. As shown in Fig. 3A,B, both motilities of the mutant strain were remarkably reduced, and those of the complemented strain were almost, although not completely, restored. To compare the amount of flagellin protein in whole-cell lysates and in the culture supernatant from each strain, immunoblot analysis probed with an anti-flagellin antibody was performed (Fig. 3C,D). The quantitative analysis demonstrated that the WT and complemented strains produced a larger amount of flagellin protein than the *vfr* mutant. To investigate the reason for the reduced production of flagellin protein in the *vfr* mutant,

Fig. 2 Inoculation of host tobacco leaves by a 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 representative results obtained from three independent experiments. (B) Bacterial population was calculated at 0, 3 and 6 days post inoculation. The bars represent standard deviations for three independent experiments. Symbols indicate a significant difference from the wild-type (WT) or complemented strain (vs. WT, * $P < 0.01$; vs. complemented strain, † $P < 0.01$).

Fig. 3 Surface motilities and flagellin production. Surface swimming motility on a 0.25% agar plate of MMMF medium (50 mM potassium phosphate buffer, 7.6 mM $(\text{NH}_4)_2\text{SO}_4$, 1.7 mM MgCl_2 and 1.7 mM NaCl , pH 5.7, supplemented with 10 mM each of mannitol and fructose) at 23 °C (A) and surface swarming motility on a 0.45% agar plate of SWM medium (0.5% peptone, 0.3% yeast extract) at 27 °C (B). Photographs show each plate after 48 h of incubation. Data shown in parentheses are the average of three independent replicates. Immunoblot analysis of flagellin protein in whole-cell lysates (ppt, C) and supernatant (sup, D) from each strain using anti-flagellin antibody (top panels). The signal intensity was quantified using ImageJ 1.45 software. The relative signal intensity in the wild-type (WT) was set to '1' (bottom panels). Symbols indicate a significant difference from the WT or complemented strain (vs. WT, * $P < 0.01$; vs. complemented strain, † $P < 0.01$).

microarray analysis was carried out according to the methods described in Experimental procedures (Table S1, see Supporting Information). The result showed that the expression of certain flagella-related genes was reduced in the vfr mutant strain (Table 1), indicating that Vfr probably positively controls flagellar gene expression, in contrast with the regulation in *P. aeruginosa* (Dasgupta *et al.*, 2002). Although the expression level of the *fliC* gene from microarray data was not changed remarkably in the vfr mutant (0.95-fold expression), other flagellar biogenesis-related genes showed lower expression than in the WT strain (Table 1). This might be the reason for the lower production of flagella filaments in the vfr mutant, although the mechanism is not yet clear (Fig. 3C,D).

Microarray analysis also revealed that the expression of T4P-related genes in the vfr mutant was weaker than in the WT strain (Table 1). Our previous results have demonstrated that T4P-defective mutants in *Pta6605* show impaired surface swimming and swarming motilities and reduced expression of the vfr gene (Taguchi and Ichinose, 2011). However, the amount of T4P from the vfr mutant strain was not significantly different from that of the WT strain in the lysate of whole cells incubated in LB with 10 mM MgCl_2 for 24 h (data not shown). We next prepared pilus-containing surface fractions from each strain grown on a KB plate. The result of Western blotting revealed that the amount of pilin was not changed remarkably in the vfr mutant (Fig. S1A, see Supporting Information). There was no significant difference in the

transcription levels of the *pilA* gene in the WT and vfr mutant (0.83-fold expression, Table 1).

Antibiotic tolerance

Recently, we have reported that the T4P-related mutant strains highly express *mexAB/oprM* genes, but show reduced expression of *vfr* and *cyaA* genes (Taguchi and Ichinose, 2011). It has been reported that the MexAB/OprM pump in *P. aeruginosa* and *E. coli* shows low specificity to substrates such as most of the common antibiotics, dyes, detergents and organic solvents (Nikaido, 2009). In the plant pathogens *Pph1448A*, *PsyB728a* and *PtoDC3000*, mutation of this RND pump reduces the tolerance to a wide range of antimicrobials, with the exception of the aminoglycosides, gentamicin, kanamycin and streptomycin (Stoitsova *et al.*, 2008). Microarray analysis revealed that the expression of the *mexAB/oprM* genes was significantly up-regulated in the vfr mutant strain (Table 1). The tolerance to antibiotics was compared by a growth inhibition test on a plate using chloramphenicol, spectinomycin, cefotaxim and crystal violet. The results displayed in Fig. 4A indicate that the vfr mutant shows a higher tolerance to these antibiotics, except for crystal violet. Enhanced expression of the *mexA* and *oprM* genes in the vfr mutant strain was also confirmed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis [Figs 4B and S2 (Supporting Information)].

Table 1 Gene expression profiles of *vfr* mutant strain compared with those of wild-type (WT) strain by microarray analysis.

Gene locus	Name	Δvfr /WT	Function of gene product	P value
Multidrug resistance				
PSPPH_4014	<i>mexB</i>	5.234	Multidrug/solvent transporter	1.19E-10
PSPPH_4012	<i>ttgR</i>	4.472	Transcriptional regulator	8.91E-03
PSPPH_4015	<i>oprM</i>	4.209	NodT family outer membrane efflux lipoprotein	8.05E-08
PSPPH_4013	<i>mexA</i>	4.038	RND family efflux transporter MFP subunit	1.27E-10
Hrp type III secretion system				
PSPPH_1265	<i>shcM</i>	0.426	Type III chaperone protein	5.95E-07
PSPPH_1274	<i>hrpB</i>	0.501	Type III secretion component protein	1.88E-05
PSPPH_1278	<i>hrpF</i>	0.501	Type III secretion component protein	3.98E-05
PSPPH_1295	<i>hrpK1</i>	0.519	Type III effector	5.13E-06
PSPPH_1272	<i>hrpA2</i>	0.567	Type III helper protein	1.29E-05
Iron uptake				
PSPPH_2756	<i>acsA</i>	0.351	Achromobactin biosynthetic protein	3.14E-06
PSPPH_2754	<i>acsC</i>	0.462	Achromobactin biosynthetic protein	1.65E-03
Flagellum biogenesis				
PSPPH_3370	<i>fliP</i>	0.475	Flagellar biosynthesis protein	2.61E-07
PSPPH_3390	<i>fliD</i>	0.531	Flagellar hook-associated protein	1.61E-07
PSPPH_3363	<i>fliA</i>	0.623	Flagellar biosynthesis sigma factor	2.11E-06
PSPPH_3371	<i>fliO</i>	0.615	Flagellar protein	1.23E-05
PSPPH_3399	<i>flgI</i>	0.634	Flagellar basal body P-ring protein	1.45E-06
Type IV pilus biogenesis				
PSPPH_0481	<i>pill</i>	0.568	Type IV pilus biogenesis protein Pill	5.30E-05
PSPPH_0480	<i>pilH</i>	0.633	Type IV pilus response regulator PilH	7.08E-05
PSPPH_0730		0.653	Type IV pilus-associated protein, putative	1.50E-04
PSPPH_0479	<i>pilG</i>	0.657	Type IV pilus response regulator PilG	7.37E-04
PSPPH_4436		0.730	General secretion pathway/type 4 pilus assembly protein	6.13E-03
Second messenger biosynthesis				
PSPPH_0905		0.32	GGDEF domain-containing putative diguanylate cyclase	1.91E-08

A *t*-test was applied to the signal intensities of the WT and each mutant, and $P < 0.05$ was considered to be statistically significant.

Fig. 4 Antibiotic tolerance. (A) The diameter of the growth inhibition zone by chloramphenicol (Cm, 175 μ g), spectinomycin (Spe, 250 μ g), cefotaxim (Cef, 250 μ g) and crystal violet (CV, 50 μ g) was measured after incubation for 24 h at 27 °C. The bars represent the standard deviations for three independent experiments. (B) Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *mexA* and *oprM* gene expression in the wild-type (WT), *vfr* mutant and complemented strains. Expression levels were normalized to the *rpoD* gene, and the level of this gene expressed in WT was set to '1'. Data are the representative results obtained from three independent experiments. Symbols indicate a significant difference from the WT or complemented strain (vs. WT, * $P < 0.01$; vs. complemented strain, † $P < 0.01$).

Hypersensitive response (HR)-inducing activity in nonhost plant

Microarray analysis also showed that the expression of several *hrp*-related genes, which encode for the Hrp type III secretion

apparatus, Hrp effectors and Hrp chaperone proteins, was decreased by approximately one-half in the *vfr* mutant strain compared with the WT strain (Table 1). It is known that the *hrp* gene cluster is required for HR in nonhost plants (Lindgren *et al.*, 1986). Therefore, we compared the cell death and callose deposition-

Fig. 5 Defence response in nonhost *Arabidopsis thaliana* leaves and *hrp* gene expression. *Arabidopsis* leaves were infiltrated with each bacterial strain at a density of 2×10^8 colony-forming units (cfu)/mL, and cell death (A) and callose deposition (B) were observed 48 h after inoculation. The average number of callose deposits with standard deviation (SD) per 4-mm² area from three independent experiments is shown in parentheses. Scale bar, 500 μ m. (C) Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *hrpK* and *hrpA* gene expression in the wild-type (WT), *vfr* mutant and complemented strains. Expression levels were normalized to the *rpoD* gene, and the level of this gene expressed in WT was set to '1'. Data are the representative results obtained from three independent experiments. Symbols indicate a significant difference from the WT or complemented strain (vs. WT, **P* < 0.01; vs. complemented strain, †*P* < 0.01).

inducing activity of each strain in the nonhost *Arabidopsis thaliana*. Trypan blue staining clearly demonstrated that the *vfr* mutant strain caused only weak cell death, and aniline blue staining indicated that the induction of callose deposition was three times lower in nonhost *A. thaliana* leaves (Fig. 5A,B). Real-time qRT-PCR analysis confirmed that the expression of the *hrpA* and *hrpK* genes was reduced in the *vfr* mutant strain (Figs 5C and S2).

Production of siderophores

Because iron is an indispensable growth factor for all organisms, including bacteria, an iron acquisition mechanism has developed using a chelator called a siderophore (Ravel and Cornelis, 2003; Smith *et al.*, 2005). The fluorescent *Pseudomonas* group produces a major siderophore compound called pyoverdine. A second siderophore, achromobactin, has been reported in *PsyB728a* and *Erwinia chrysanthemi* (Berti and Thomas, 2009; Franza *et al.*, 2005). A chrome azurol-S (CAS) assay (Schwyn and Neilands, 1987) revealed that siderophore production of the *vfr* mutant was reduced (Fig. 6A). As there was no significant difference in the amount of pyoverdine in the culture supernatant of each strain (data not shown), reduced iron acquisition in the *vfr* mutant might be caused by decreased expression of achromobactin-related genes. Our microarray analysis and qRT-PCR revealed that the *vfr* mutant showed reduced expression of *acsA* and *acsC* genes for achromobactin biosynthetic enzymes (Table 1, Figs 6B and S2).

Acylhomoserine lactone (AHL) production and biofilm formation

We compared the amounts of AHLs extracted from each bacterial culture with ethyl acetate using the biosensor *Chromobacterium violaceum* CV026; however, there was no significant difference in AHL production (Fig. S1B). To examine whether Vfr is involved in biofilm formation, biofilm formation on a polystyrene tube after 48 h of incubation was investigated. As shown in Fig. S1C, the ability of the *vfr* mutant strain to form a biofilm was similar to that of the WT. These results are different from those found in *P. aeruginosa*, where AHL production is controlled by Vfr (Whitchurch *et al.*, 2005), and suggest that biofilm formation and AHL production are not under the regulation of Vfr in *Pta6605*.

Construction of *cyaA* mutant and measurement of cAMP

In *P. aeruginosa*, it has been reported that cAMP is required for Vfr-dependent regulation, except for the *LasR* promoter (Fuchs *et al.*, 2010). To examine the effect of cAMP on the control of the expression of virulence genes in *Pta6605*, a mutation was introduced into a gene for adenylate cyclase. Although two genes, *cyaA* and *cyaB*, for adenylate cyclase are known in *P. aeruginosa* (Wolfgang *et al.*, 2003), we found only *cyaA* in the genome of *Pph1448A*. Therefore, *cyaA* in *Pta6605* was disrupted (Fig. 7A). To

Fig. 6 Siderophore production. (A) Quantitative analysis of siderophore production. The bars represent the standard deviations for three independent experiments. CAS, chrome azurol-S; OD₆₃₀, optical density at 630 nm. (B) Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *acsA* and *acsC* gene expression in the wild-type (WT), *vfr* mutant and complemented strains. Expression levels were normalized to the *rpoD* gene, and the level of this gene expressed in WT was set to '1'. Data are the representative results obtained from three independent experiments. Symbols indicate a significant difference from the WT or complemented strain (vs. WT, **P* < 0.01; vs. complemented strain, †*P* < 0.01).

analyse the effect of the *vfr* or *cyaA* mutation on the cAMP level, we extracted cAMP from the KB liquid culture of each bacterium. The *cyaA* mutant almost completely abolished cAMP production, and its complemented strain partially restored it (Fig. 7B). This result confirms that CyaA plays a major role in cAMP biosynthesis in *Pta6605*. The concentration of cAMP in the *vfr* mutant was also reduced compared with the WT and complemented strain (Fig. 7B), although the microarray analysis detected no significant difference in the transcription level of *cyaA* (1.08-fold expression, Table S1).

Phenotype of *cyaA* mutant

The phenotype of the *cyaA* mutant was compared with that of the *vfr* mutant; the *cyaA* mutant showed reduced virulence on its host tobacco plant (Fig. 8A), decreased surface swimming and swarming motilities (Fig. 8B), reduced amount of flagellin protein (Fig. 8C), decreased sensitivity to chloramphenicol, spectinomycin and cefotaxim (Fig. 8D), reduced ability to induce defence responses, such as HR cell death (Fig. 8E), and decreased callose deposition (Fig. 8F) in nonhost *A. thaliana* leaves. The complemented strain recovered well the ability to induce defence responses (Fig. 8E,F) and partially restored virulence (Fig. 8A), surface motilities (Fig. 8B) and flagellin production (Fig. 8C), but did not recover the sensitivity to antimicrobial compounds (Fig. 8D). Furthermore, there was no significant difference in the production of AHL and pilin between the *cyaA* mutant and WT strain, as found in the *vfr* mutant (data not shown). Thus, this study shows that the phenotypes of *cyaA* and *vfr* mutants resemble each other, suggesting that Vfr regulates the expression of virulence genes with a cAMP-dependent mechanism in *Pta6605*.

DISCUSSION

In this study, we investigated the role of Vfr, a member of the Crp family, in the virulence of the phytopathogenic bacterium *Pta6605*. Although Crp is the best-known transcriptional activator and regulates the transcription of about 100 genes in *E. coli* (Fic *et al.*, 2009), the role of Crp in phytopathogenic bacteria is not clear. The binding signal to cAMP might be transduced from the N-terminal cAMP-binding domain to the C-terminal domain with a conserved helix-turn-helix motif for DNA binding. The substitutional analysis of Crp mutants at amino acid positions 83, 127 and 128 confirmed the importance of these residues in cAMP-mediated Crp activation (Lee *et al.*, 1994). Sequence analysis of the *vfr* gene in *Pta6605* revealed that both the predicted cAMP- and DNA-binding sites are conserved, although T132 in *Pta6605* replaces serine in the Crp of *E. coli* (Fig. 1B). Very recently, Cordes *et al.* (2011) have reported the crystal structure of full-length Vfr and an N-terminal-truncated Vfr-Ndel7 in *P. aeruginosa*. They demonstrated that Vfr is a winged-helix DNA-binding protein and that the N-terminal region of Vfr plays a unique role as a second ligand-binding site. The residues E57–M61 and Q175–R185 in the helix-turn-helix motif form the secondary ligand-binding pocket in Vfr, but these regions are not well conserved in Crp. The equivalent residues, R59 and R179, in *Pta6605* are also conserved, suggesting that the N-terminal region and helix-turn-helix motif might also be important for a second ligand-binding site in *Pta6605*.

Lee *et al.* (1994) suggested that T128 in Crp in *E. coli* might have an ability to bind to a cyclic guanosine monophosphate (cGMP). Furthermore, a recent study on Clp in *Xcc* has demonstrated that E99 (corresponding to residue S83 in Crp and Vfr) is essential for binding with the cyclic di-GMP (Tao *et al.*, 2010). Cyclic di-GMP is a ubiquitous second messenger synthesized by

Fig. 7 Generation of *cyaA* mutant in *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta6605*) and quantification of cyclic 3',5'-adenosine monophosphate (cAMP). (A) Schematic organization of the *cyaA* gene in *Pseudomonas syringae* pv. *phaseolicola* 1448A (*Pph1448A*) and construction of the *cyaA* mutant in the corresponding region of *Pta6605*. Locus IDs for *Pph1448A* are shown inside the respective arrows. Polymerase chain reaction (PCR) was carried out to isolate the *cyaA*-containing DNA fragment using primers (*cyaA-F* and *cyaA-R*), as indicated by arrowheads; the internal region was then excised by digestion with *AgeI* and *StuI*. (B) Quantification of cAMP. Each strain was grown in KB liquid medium (2% proteose peptone No.3, 8.6 mM K_2HPO_4 , 1.6 mM $MgSO_4$, 1% glycerol) for 24 h at 27 °C, and the cAMP levels were measured using a cAMP enzyme immunoassay (EIA) kit. The bars represent standard deviations for three independent experiments. Symbols indicate a significant difference from the wild-type (WT) or complemented strain (vs. WT, * $P < 0.01$; vs. complemented strain, † $P < 0.01$).

diguanylate cyclase, and plays many roles, such as the regulation of cell aggregation, motility, biofilm formation, extracellular protease production and quorum sensing, in a wide variety of bacteria (Hengge, 2009). Very recently, it has been reported that the putative diguanylate cyclase XCC2731 in *Xcc* is involved in full virulence and is regulated positively by Clp (Hsiao *et al.*, 2011). As *Pta6605* Vfr conserves S83 and T128, we could not rule out the possibility that Vfr in *Pta6605* binds to cGMP or cyclic di-GMP. Indeed, our microarray analysis revealed that the expression of a PSPPH0905 orthologue (putative diguanylate cyclase gene) was down-regulated significantly in the *vfr* mutant (0.32-fold expression, Table 1), although the role of this protein has not yet been elucidated.

Similar to the *clp* mutant in *Xcc* (Lee *et al.*, 2003), the *vfr* mutant in *Pta6605* showed lower flagella biogenesis and surface motilities (Fig. 3). Previously, we have revealed that surface swimming and swarming motilities require flagella and T4P (Shimizu *et al.*, 2003; Taguchi and Ichinose, 2011). Because the *vfr* mutant retained the swimming motility in liquid medium at the same level as the WT strain, and slowly recovered surface swimming motility with longer incubation on 0.25% agar MMMF plates (Fig. S3, see Supporting Information), reduced surface motility of the *vfr* mutant might be a result of the reduced expression of T4P-related genes (Table 1).

It has been reported that Vfr in *P. aeruginosa* negatively regulates the expression of the *fleQ* gene, which encodes the master flagellar transcriptional activator (Dasgupta *et al.*, 2002), whereas the expression of the *fleQ* gene is not changed remarkably in microarray analysis (1.37-fold expression) of WT and the *vfr* mutant in *Pta6605*. Although consensus sequences for the Vfr-binding sites (AnWWTGnGAWnYAGWTCACAT and CGTCGAT-AGTCTGACACTAGG) have been proposed by Kanack *et al.* (2006) and Fuchs *et al.* (2010), respectively, there are no identical sequences in the promoter regions of *mexA*, *acsC*, *hrpA2*, *fliD* and *pilG* in *Pph1448A*, a strain closely related to *Pta6605*.

The *vfr* mutant showed reduced HR-inducing ability in nonhost *Arabidopsis* leaves and enhanced expression of *mexAB/oprM* genes (Figs 4 and 5). Previously, we have demonstrated a similar phenotype in T4P-defective mutants: T4P-defective mutants showed reduced surface motility, decreased expression of T3SS-related genes, reduced HR, decreased virulence towards the host tobacco plant and enhanced expression of *mexAB/oprM*. Furthermore, T4P-defective mutants showed reduced expression of *vfr* and *cyaA* (Taguchi and Ichinose, 2011). Together with the results obtained in this study, our observations indicate that Vfr plays a crucial role as a global regulator of T3SS-mediated HR, virulence and MexAB/OprM-mediated tolerance to antimicrobial compounds. He *et al.* (2007) conducted microarray analysis using the *clp* mutant in *Xcc* to identify Clp-regulated genes, and found that the genes for extracellular enzymes, the biosynthesis of extracellular polysaccharide (EPS), multidrug resistance, flagellar biogenesis, Hrp system, iron uptake and protein metabolism are regulated by Clp. However, in contrast with our results, the expression of Hrp-related genes is negatively regulated by Clp and the *mexAB/oprM* genes are down-regulated in the *clp* mutant strain, suggesting that there are striking differences in the regulation of Clp in *Xcc* and Vfr in *Pta6605*.

Iron is one of the essential factors for bacterial growth, and effective iron uptake systems using a siderophore as a high-affinity iron chelator have therefore developed in pathogenic bacteria. In *Pta6605*, the siderophore pyoverdine is an intrinsic virulence factor in host tobacco infection (Taguchi *et al.*, 2010a). Our previous study demonstrated that a *pvdL*-defective mutant in *Pta6605*, which is unable to produce pyoverdine chromophores,

Fig. 8 Phenotypes of the *cyaA* mutant. (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 representative results obtained from three independent experiments. (B) Surface swimming motility at 23 °C (top panel) and surface swarming motility at 27 °C (bottom panel). Photographs show each plate after 48 h of incubation. Data shown in parentheses are the average of three independent replicates. (C) Immunoblot analysis of flagellin protein in whole-cell lysates using anti-flagellin antibody (top panels). The relative signal intensity in the wild-type (WT) was set to '1' (bottom panels). (D) Antibiotic tolerance. The diameter of the growth inhibition zone by chloramphenicol (Cm, 175 μg), spectinomycin (Spe, 250 μg) and cefotaxim (Cef, 250 μg) was measured after incubation for 24 h at 27 °C. The bars represent standard deviations for three independent experiments. *Arabidopsis* leaves were infiltrated with each bacterial strain at a density of 2×10^8 cfu/mL, and cell death (E) and callose deposition (F) were observed 48 h after inoculation. The average number of callose deposits with standard deviation (SD) per 4-mm² area from three independent experiments is shown in parentheses. Symbols indicate a significant difference from the WT or complemented strain (vs. WT, * $P < 0.01$; vs. complemented strain, † $P < 0.01$).

shows a reduced ability to produce tabtoxin, EPS and AHL, and exhibits significantly reduced virulence on host tobacco leaves (Taguchi *et al.*, 2010a). Moreover, the AHL synthase-defective mutant $\Delta psyl$ shows reduced ability to produce the siderophore pyoverdine (Taguchi *et al.*, 2006a). Our microarray results in this study indicated that the *vfr* mutant in *Pta6605* showed reduced ability to acquire iron as a result of the lower biosynthesis of achromobactin, a second siderophore found in *PsyB728a* (Berti and Thomas, 2009). Because the amount of AHL produced by the *vfr* mutant was similar to that of the WT strain, the production of achromobactin must be regulated under Vfr, not under a quorum sensing system (Figs 6 and S1B). In *E. chrysanthemi*, not only the catechol-type siderophore chrysobactin, but also achromobactin, is necessary for full virulence in plant infection (Franza *et al.*, 2005). *Erwinia chrysanthemi* might acquire iron by achromobactin at an early stage in infection when growing as an epiphyte, and then shift to using chrysobactin after infection has been established. Thus, *E. chrysanthemi* uses each siderophore at a different stage of infection. In *PsyB728a*, it has also been shown that pyoverdine is not produced in the epiphytic growing stage in plants (Berti and Thomas, 2009). Taking these results together, we speculate that AHL and Vfr provide independent regulatory systems for the production of the different siderophores, pyoverdine and achromobactin, involved in iron uptake in *Pta6605*.

Fulcher *et al.* (2010) reported that the concentration of intracellular cAMP was reduced in the *vfr*, *pilG* and *pilH* mutants of *P. aeruginosa*, although the protein level of the adenylate cyclase, CyaB, was not affected. PilG and PilH are members of the Chp chemotaxis-like chemosensory system, and are known to regulate pilus function via a two-component signalling system. These results suggest that the Chp system regulates the cAMP level by modulating CyaB activity. In *Pta6605*, we found that the cAMP level in the *vfr* mutant decreased (Fig. 7B), but the expression of *cyaA* did not change (1.08-fold expression, Table S1). Furthermore, our microarray analysis revealed that the expression levels of *pilG* and *pilH* were reduced (0.66- and 0.63-fold expression, respectively, Table 1). Although the role of the Chp system in *Pta6605* has not yet been clarified, Vfr might also be a positive regulator of the Chp system and control cAMP biosynthesis in *Pta6605*.

In this study, we have demonstrated that Vfr is a crucial global regulator for motility, flagellar biogenesis, multidrug resistance, iron uptake and T3SS using the quorum sensing-independent mechanism in *Pta6605*. These results, together with those obtained using T4P-defective mutants (Taguchi and Ichinose, 2011), show that there is a global regulatory system of cAMP, Vfr and T4P in this bacterium (Fig. 9). Vfr is required for virulence, T3SS, production of achromobactin and induction of defence responses, and suppresses MexAB/OprM-mediated multidrug resistance. The T4P-defective mutant shows decreased expression of *vfr* and *cyaA*, and similar phenotypes, except for biofilm formation, to the *vfr* and *cyaA* mutants (Taguchi and Ichinose, 2011),

Fig. 9 Schematic depiction of virulence factor regulator (Vfr)- or quorum sensing (QS)-mediated regulatory network of virulence expression in *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta6605*). Dotted arrows are not yet confirmed. AHL, acylhomoserine lactone; cAMP, cyclic 3',5'-adenosine monophosphate; EPS, extracellular polysaccharide; MDR, multidrug resistance; T3SS, type III secretion system; T4P, type IV pili.

suggesting that biofilm formation requires T4P, but not Vfr and CyaA. These results may indicate that T4P regulates the function of Vfr and CyaA. However, Vfr might affect the function of T4P because the expression of T4P-related genes is lower in the *vfr* mutant. On the other hand, the results using flagella-mediated motility-defective mutants, AHL production-defective mutants and pyoverdine synthesis-defective mutants demonstrated another crucial regulatory network of virulence controlled by AHL (Taguchi *et al.*, 2006a, 2010a, 2010b). Therefore, *Pta6605* might have evolved intricate regulatory networks for virulence through two different surface appendages, flagella and T4P. To confirm this, we plan to analyse the promoters of Vfr target genes in a future study.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 2. The *Pta6605* strains were maintained as described by Taguchi *et al.* (2006b). The *E. coli* strains were grown at 37 °C in LB medium. *Chromobacterium violaceum* CV026 was grown at 30 °C in LB medium with a final concentration of 50 µg/mL kanamycin (McClean *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 11 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 colony-forming units (cfu)/mL. The inoculated leaf discs were incubated on 3 MM paper (Whatman Plc, Brentford, UK) with a

Table 2 Bacterial strains and plasmids.

Bacterial strain/plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i> DH5 α	<i>F</i> ⁻ λ - <i>o80dLacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1endA1 hsdR17</i> (rK-mK+) <i>supE44 thi-1 gyrA relA1</i>	Takara, Kyoto, Japan
S17-1	<i>thi pro hsdR hsdR hsdM</i> ⁺ <i>recA</i> (<i>chr::RP4-2-Tc::Mu-Km::Tn7</i>)	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	Wild-type isolated from tobacco, Nal ^r	Shimizu <i>et al.</i> (2003)
6605- <i>vfr</i>	Isolate 6605 <i>vfr</i> , Nal ^r	This study
6605- <i>cyaA</i>	Isolate 6605 <i>cyaA</i> , Nal ^r	This study
6605- <i>vfr</i> (pBSL118 <i>vfr</i>)	Isolate 6605 <i>vfr</i> , Nal ^r (pBSL- <i>vfr</i>)	This study
6605- <i>cyaA</i> (pBSL118 <i>cyaA</i>)	Isolate 6605 <i>cyaA</i> , Nal ^r (pBSL- <i>cyaA</i>)	This study
Plasmids		
pGEM-T Easy	Cloning vector, Amp ^r	Promega, Tokyo, Japan
pGEM- <i>vfr</i>	<i>vfr</i> -containing 1984-bp DNA fragment in pGEM-TEasy	This study
pCR-BluntII-TOPO	Cloning vector, Km ^r	Invitrogen, Carlsbad, CA, USA
pCR-BluntII-TOPO- <i>cyaA</i>	<i>cyaA</i> -containing 3415-bp DNA fragment in pCR-BluntII-TOPO	This study
pK18 <i>mob</i> SacB	Small mobilizable vector, Km ^r , sucrose sensitive (<i>sacB</i>)	Schäfer <i>et al.</i> (1994)
pK18- <i>vfr</i>	Defective <i>vfr</i> -containing DNA fragment in pK18 <i>mob</i> SacB	This study
pK18- <i>cyaA</i>	Defective <i>cyaA</i> -containing DNA fragment in pK18 <i>mob</i> SacB	This study
pBSL118	Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp ^r , Km ^r	Alexeyev <i>et al.</i> (1995)
pBSL- <i>vfr</i>	<i>vfr</i> -containing 1984-bp DNA fragment in pBSL118	This study
pBSL- <i>cyaA</i>	<i>cyaA</i> -containing 3415-bp DNA fragment in pBSL118	This study

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

distilled water supply. The bacterial population from inoculated leaves was determined as described by Taguchi and Ichinose (2011).

Arabidopsis thaliana ecotype Columbia-0 was grown at 22 °C with a 16-h photoperiod. To detect HR induction and callose deposition, *Arabidopsis* leaves (three leaves for each strain) were infiltrated with bacterial suspension in 10 mM MgSO₄ without Silwet at a density of 2×10^8 cfu/mL.

Construction of mutant and its complemented strain

To generate the *vfr*-defective mutant, the genetic region including *vfr* was isolated by a pGEM-T-Easy vector cloning system (Promega, Madison, WI, USA) using a set of PCR primers (*vfr*-F and *vfr*-R, Table 3) that were designed on the basis of the registered sequence of the *vfr* gene of *Pph1448A* (accession number, NC_005773, Fig. 1). For the construction of the *cyaA*-defective mutant, the genetic region including *cyaA* was cloned in a pCR-BluntII-TOPO vector (Invitrogen, Carlsbad, CA, USA) using a set of PCR primers (*cyaA*-F and *cyaA*-R). The recombinant plasmids obtained were digested with *NarI* and *BsmFI* to delete the internal 53-bp region for the generation of the *vfr* mutant plasmid, and with *StuI* and *AgeI* to delete the internal 1071-bp region of the *cyaA* mutant plasmid. After digestion, 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*mob*SacB 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 changed phenotype originated in the corresponding mutation, each *vfr* or *cyaA* mutant strain was complemented using the PCR product described above, because the predicted promoter and entire open reading frame

Table 3 Primer sequences used in this study.

Primer name	Sequence (5'–3')
<i>vfr</i> -F	TGCAAGACTCGATCATCGAC
<i>vfr</i> -R	CATAATCGTGAGTGATCTCC
<i>cyaA</i> -F	TTATCTGACCAAGCCTCGGGC
<i>cyaA</i> -R	CAATGGGCTCGGCGTTGCTG
<i>rpoD</i> -F	GATCGTCTCGATCATGTGCA
<i>rpoD</i> -R	ATTGGTCGTCTCGAGCTGA
<i>proC</i> -F	GAGGCCACGCAATTCAGTC
<i>proC</i> -R	GATCAAGGGCGCAGATGTCG
<i>hrpK</i> -F	AGCATCGTGAATCAACCCAC
<i>hrpK</i> -R1	CTGCGAGTCAGCAGACTCAT
<i>acsA</i> -F	ATGAACCTCACTTCACTCGC
<i>acsA</i> -R	CCCTTTTCATGAACCTTCC
<i>acsC</i> -F	GCTGAAACGCAACACCGTTT
<i>acsC</i> -R	CTCAACGCCAGATGCAAATC
<i>hrpA</i> -F	TGAGTTCTTGACTAACGCG
<i>hrpA</i> -R	GAAATCAGAACTGGACGACC
<i>mexA</i> -F	TTGAGACAACAGGCTCTGCG
<i>mexA</i> -R	GTCCACGTAGATTGGGTGCGA
<i>oprM</i> -F	GTCCACGTAGATTGGGTGCGA
<i>oprM</i> -R	GAAACACCGACTTCATTGCT

were contained in this region. The resultant plasmid was ligated with pBSL118, a transposon vector (Alexeyev *et al.*, 1995), at the *Apal* site to generate pBSL-*vfr* and at the *EcoRI* site to generate pBSL-*cyaA*, and was introduced into the respective mutant by conjugation using the *E. coli* S17-1 λ pir strain to generate the complemented strains *vfr*-C and *cyaA*-C. The primers used in this study are listed in Table 3. The DNA sequences of the isolated clone and deletion mutants were determined (ABI PRISM 310, Applied Biosystems, Chiba, Japan) using a BIG Dye terminator cycle sequencing kit.

Motility assay

Motility experiments were performed as described previously (Taguchi *et al.*, 2006b, 2010b). The surface swimming motility was observed in 0.25% agar MMMF plates after 48, 72 and 96 h of incubation at 23 °C, whereas the surface swarming motility was assessed in 0.45% agar SWM plates after 48 h of incubation at 27 °C. For the quantification of motilities, the diameters of the swimming and swarming areas were measured after inoculation for 48 h.

CAS assay

The siderophore production in each bacterial culture supernatant was determined by the methods of Schwyn and Neilands (1987), as described previously (Taguchi *et al.*, 2006a).

Pyoverdine measurement

The concentration of pyoverdine was estimated spectrophotometrically as the absorbance at 405 nm using the supernatant of the MMMF culture medium of each strain after incubation for 24 h at 25 °C.

Preparation of pilus-containing surface fraction

Pili were partially purified as described previously (Taguchi and Ichinose, 2011) with a minor modification. Each bacterium was grown on KB plates for 72 h at 27 °C. Cells were scraped off, resuspended in 50 mM sodium phosphate buffer (pH 7.0) and adjusted to an optical density at 600 nm (OD_{600}) of 1.0. Pili were sheared off the cells by stirring for 30 min, and the cells were removed by centrifugation at 10 000 *g* for 20 min. After filtration of the supernatant through a 0.45- μ m pore filter, polyethylene glycol (PEG6000) and NaCl were added to final concentrations of 4% and 0.5 M, respectively. Pili-containing fractions were obtained as pellets by centrifugation at 100 000 *g* for 30 min, and were resuspended in 500 μ L of water.

Tolerance to antibiotics

Antibiotic tolerance was assessed using paper discs (3 MM paper) containing 10 μ L of chloramphenicol (17.5 μ g/ μ L), spectinomycin (25 μ g/ μ L), cefotaxim (25 μ g/ μ L) and crystal violet (0.5%), as described previously (Taguchi *et al.*, 2006a).

Biofilm formation

The detection of biofilms was performed as described previously (Taguchi and Ichinose, 2011).

RNA preparation, microarray analysis and real-time qRT-PCR

Each bacterial strain was grown in LB medium with 10 mM $MgCl_2$ at 27 °C to an OD_{600} of 0.3. After centrifugation, the cells were further incubated in MMMF medium for 1 h. Total RNA was then extracted using a High Pure RNA Isolation kit (Roche, Mannheim, Germany) according to the manu-

facturer'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).

For real-time qRT-PCR, 2 μ g of total RNA were used to synthesize cDNA with AMV RTase in a total of 50 μ L of reaction mixture, according to the manufacturer's instructions (Takara). Specific primer sets for *mexA*, *oprM*, *hrpA*, *hrpK*, *acsC* and *acsA* were designed on the basis of the sequence of *Pph1448A*. Real-time qRT-PCR was carried out using a Rotor-Gene 300™ (Corbett Research, Sydney, Australia) and SYBR premix Ex Taq™ (Takara), with primers at a final concentration of 0.2 μ M each and 1 μ L of cDNA product as a template in a 25- μ L reaction mixture. PCR was performed with one denaturation cycle of 20 s at 95 °C and 45 cycles of 15 s at 55 °C, 15 s at 72 °C and 30 s at 95 °C. The expression of each gene was normalized by *rpoD* and *proC*, which encode RNA polymerase sigma factor and pyrroline-5-carboxylate reductase, respectively, as housekeeping genes (Kanda *et al.*, 2011; Savli *et al.*, 2003). Melting curve analysis confirmed that each PCR was a single product. The PCRs were performed with a high linearity. The *E* value as primer efficiency was calculated from the slope of each standard curve equation [$E = 10(-1/\text{slope}) - 1$] (Michán and Pueyo, 2009). Each primer pair showed optimal primer efficiency (data not shown). The data were analysed using the $\Delta\Delta C_T$ (cycle threshold) method.

Detection of AHLs

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

Detection of cell death and callose deposition assay

Cell death and callose deposition were detected by lactophenol trypan blue and aniline blue staining, respectively, as described previously (Naito *et al.*, 2007; Taguchi and Ichinose, 2011). For quantitative analysis, the number of callose deposits per 4-mm² area was counted, and its average was calculated.

Immunoblot analysis

Immunoblot analysis was carried out as described previously (Taguchi *et al.*, 2006b). For the detection of flagellin protein in whole-cell lysates, an overnight culture of each bacterium (equivalent to 100 μ L of bacterial culture) in LB with 10 mM $MgCl_2$ at 27 °C was centrifuged. For the preparation of total protein, the bacterial suspensions cultured overnight in MMMF medium were precipitated by the addition of one-fifth volume of 10% trichloroacetic acid and dissolved in a sample buffer. After separation on 12.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, 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, Ipswich, 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).

For the detection of pilin protein, immunoblot analysis was performed using a rabbit polyclonal anti-PilA-specific peptide antibody (peptide sequence: VAENYSNGSTQADVC), according to the method reported previously (Taguchi and Ichinose, 2011).

cAMP assay

Each bacterial strain was grown in KB liquid medium for 24 h, and the concentration was adjusted to a density of 6×10^8 cfu/mL with 1 mL of PBS (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl, 1.47 mM KH_2PO_4 , pH 7.4). Each bacterial solution was frozen at -80°C , and then thawed at room temperature and sonicated for 5 min on ice. After centrifugation at 15 000 g for 5 min, 50 μL of supernatant were used to measure cAMP, according to the manufacturer's instructions (Cyclic AMP EIA Kit, Cayman Chemical Co., Ann Arbor, MI, USA).

Statistical analysis

The results of bacterial populations in host tobacco leaves, motilities, flagellin proteins detected by Western blot, growth inhibition assay, real-time qRT-PCR, callose deposition assay, CAS assay and cAMP 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, mutant and complemented strains. $P < 0.01$ was considered to be statistically significant.

Nucleotide sequence accession numbers

The nucleotide sequences of the *vfr* and *cyaA* genes have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers AB674327 and AB733003, respectively.

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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 Detection of pilin protein and acylhomoserine lactones (AHLs) and biofilm formation in wild-type (WT), *vfr* mutant and complemented strain. (A) Pilus-containing surface fractions were analysed using an anti-pilin-specific antibody. (B) AHLs, as *N*-hexanoyl-L-homoserine lactone (HHL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), were visualized using violacein production by *Chromobacterium violaceum* CV026. (C) Biofilm production. Each bacterium was grown in Luria–Bertani (LB) medium with 10 mM MgCl₂, transferred to fresh MMMF medium [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] and incubated for 48 h at 25 °C without agitation. Adherent bacteria were stained with 0.5% crystal violet, and washed with water. All photographs show representative results obtained from two or three independent experiments.

Fig. S2 Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *hrpA*, *hrpK*, *acsA*, *acsC*, *mexA* and *oprM* gene expression in the wild-type (WT), *vfr* mutant and complemented strains. The expression level of each gene was normalized to that of the *proC* gene, and the relative value of WT expression was set to '1' as a control. Data are representative results obtained from three independent experiments. Symbols indicate a significant difference from the WT or complemented strain (vs. WT, **P* < 0.01; vs. complemented strain, †*P* < 0.01).

Fig. S3 Surface swimming motility on a 0.25% agar plate of MMMF medium [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] at 23 °C of wild-type (WT), *vfr* mutant and complemented strain. Photographs show each plate after 72 and 96 h of incubation.

Table S1 Gene expression profiles of *vfr* mutant strain compared with the wild-type (WT) strain by microarray analysis.