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Gac two-component system in Pseudomonas syringae pv. tabaci is required for virulence but not for hypersensitive reaction

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i) Title: **Gac two-component system in *Pseudomonas syringae* pv. *tabaci* is required for virulence but not for hypersensitive reaction**

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vii) Abbreviations: AHLs, *N*-acyl homoserine lactones; HR, hypersensitive reaction; HSL, homoserine lacton

Abstract

Pseudomonas syringae pv. *tabaci* 6605 causes wildfire disease on host tobacco plants. To investigate the regulatory mechanism of the expression of virulence, Gac two-component system-defective mutants, Δ*gacA* and Δ*gacS*, and a double mutant, Δ*gacA*Δ*gacS*, were generated. These mutants produced smaller amounts of *N*-acyl homoserine lactones, required for quorum sensing, had lost swarming motility, and had reduced expression of virulence-related *hrp* genes and the *algT* gene required for exopolysaccharide production. The ability of the mutants to cause disease symptoms in their host tobacco plant was remarkably reduced, while they retained the ability to induce hypersensitive reaction (HR) in non-host plants. These results indicated that the Gac two-component system of *P. syringae* pv. *tabaci* 6605 is indispensable for virulence on the host plant, but not for HR induction in non-host plants.

Introduction

Bacteria have the ability to adapt to environmental conditions and to regulate gene expression, including virulence-related genes. Bacteria perceive environmental signals or cues by using signal sensor kinases and transduce the information to transcription factors by two-component systems. The sensor kinase GacS autophosphorylates and activates its partner, the response regulator GacA, and together they constitute a Gac two-component system. Both GacS and GacA are present in a wide variety of bacteria (Heeb and Haas 2001). For example, about 20 GacS and GacA homologs have been identified in many Gram-negative bacteria, including *E. coli, Salmonella enterica*, *Erwinia carotovora*, *Pseudomonas, Vibrio*, and *Azotobacter vinelandii*.

The Gac two-component system has been found to regulate an array of phenotypes, including regulation of secondary metabolites such as quorum sensing molecules (Chancey et al. 1999), biofilm formation (Parkins et al. 2001), alginate biosynthesis (Willis et al. 2001), production of toxins (Barta et al. 1992; Kitten et al. 1998), proteases (Barta et al. 1992), siderophores (Stintzi et al. 1998), and swarming motility (Kinscherf and Willis 1999).

Quorum-sensing is the cell-density-dependent regulation of specific gene expression in response to extracellular chemical signals produced by the bacteria themselves (Fuqua et al.

2001). In Gram-negative bacteria, such signal molecules are usually *N*-acyl homoserine lactones (AHLs), which differ in the structure of their *N*-acyl side chains (Elasri et al. 2001). Quorum sensing regulates epiphytic populations, production of extracellular polysaccharides (EPSs), and motility and virulence in *Pseudomonas syringae* pv. *syringae* B728 (Quinones et al. 2004, 2005). The Gac two-component system positively regulates the quorum sensing signals in *P. syringae* pv. *syringae* (Kinscherf and Willis 1999; Kitten et al. 1998), *P. syringae* pv. *tomato* DC3000 (Chatterjee et al. 2003), and *P. aureofaciens* (Chancey et al. 1999). Furthermore, in *P. aeruginosa*, quorum-sensing is required for production and secretion of multiple extracellular virulence factors, such as proteases and toxins, and biofilm maturation (Parkins et al. 2001). A biofilm is a complex of bacterial cells embedded in a complex of highly heterogeneous EPSs that attach to a host surface (Costerton et al. 1995). *P. syringae* pathovars produce alginate as an EPS molecule (Fett et al. 1989). It is known that alginate production is controlled by the Gac two-component system in *P. syringae* pv. *syringae* B728a (Willis et al. 2001) and *Azotobacter vinelandii* (Castaneda et al. 2001). Alginate is an important contributing factor in successful infection of plant hosts by *P. syringae* pv. *syringae* (Yu et al. 1999).

Fluorescent *Pseudomonas* species are known to produce Fe(III)-chelating siderophores

termed pyoverdins that fluoresce under UV light (Bultreys et al. 2003). Bacteria often live in iron-deficient environments. In response to these conditions, bacteria produce siderophores to effectively acquire iron. Bacteria that are unable to use these siderophores cannot ingest iron. It has been reported that siderophore biosynthesis in *P. aeruginosa* is controlled by a quorum-sensing system (Stintzi et al. 1998).

It is also reported that a Gac two-component system regulates swarming motility in *P. syringae* pv. *tomato* DC3000 and pv. *syringae* (Chatterjee et al. 2003; Kinscherf and Willis 1999). We found that swarming motility is also required for bacterial virulence in *P. syringae* pv. *tabaci* 6605, a causal agent of bacterial wildfire disease of tobacco, using swarming-defective mutants of this bacterium (Taguchi et al. 2006b). We also found that a type-three secretion system (TTSS) is required for virulence (Marutani et al. 2005). However, how the expression of these virulence-related genes are regulated is still obscure. Here we report that the Gac two-component system regulates pleiotropic phenotypes, such as production of quorum-sensing signals and expression of *hrp* genes and other virulence factors, and is required for bacterial virulence using different Gac-defective mutants. In this study, we discuss the role of the Gac two-component system in virulence and HR induction of *P. syringae* pv. *tabaci* 6605.

Materials and methods

Pseudomonas syringae and generation of Δ*gacS*, Δ*gacA* defective mutants

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *P. syringae* strains were grown as described previously (Ishiga et al. 2005). For use as inocula, the concentration of *P. syringae* was adjusted with 10 mM $MgSO_4$.

To generate the *gacS* and *gacS/gacA* double mutants of *P. syringae* pv. *tabaci* 6605, PCR was carried out using genomic DNA and primers for *gacS* (gacS1 and gacS2) as shown in Fig. 1A. All primer sequences used in this study are listed in the supplemental Table. The nucleotide sequence of the amplified DNA fragment (2,757 bp) was cloned into a pGEM-T-Easy vector (Promega, Madison, WI, USA) to produce pGEMgacS1, which was confirmed to contain the *gacS* gene. Using pGEMgacS1 as a template DNA, PCR was carried out with a set of primers, gacS1 and gacS3, and the resulting 2,320 bp fragment was cloned into a pGEM-T-Easy vector to produce pGEMgacS2. The 5' and 3' ends of the *gacS* gene (1,131 bp and 829 bp, respectively) were prepared by digestion of pGEMgacS2 with *Sph*I/*Sal*I, and inserted into the mobilizable cloning vector pK18*mob*SacB (Schaefer et al. 1994). The resultant plasmid, pMgacS, possessed the internally truncated *gacS* gene. Thus,

6605-Δ*gacS* defective mutants were obtained by introducing pMgacS into *P. syringae* pv. *tabaci* 6605, a wild-type strain, via the *E. coli* S17-1 strain by conjugation and subsequent recombination and excision of the plasmid on KB agar containing 10% sucrose.

Similarly, PCR was also carried out using primers for *gacA* (gacA1 and gacA2) to obtain the *gacA* and *uvrC* genes together with their surrounding regions, as shown in Fig. 1B. To generate Δ*gacA* and Δ*gacS*Δ*gacA* double mutants, approximately 0.8-0.9 kb fragments located on each side of the *gacA* gene were amplified by PCR with gacA1 and gacA3 for upstream (792 bp) and gacA2 and gacA4 for downstream (890 bp) regions of *gacA*, respectively. Each amplified DNA fragment was cloned into a pGEM-T-Easy vector to obtain pGEMgacA1 and pGEMgacA2. The DNA fragments obtained by the digestion of plasmid pGEMgacA1 with *Eco*RI/*Bam*HI and by digestion of pGEMgacA2 with *Bam*HI/*Sph*I were inserted into the pK18*mob*SacB. The resulting plasmid, pMgacA, contained the genomic DNA flanking *gacA*, but lacked *gacA* itself. After confirmation of the mutated nucleotide sequence, pMgacA was introduced into the *P. syringae* pv. *tabaci* wild-type or Δ*gacS* defective mutant via *E. coli* S17-1 by conjugation. After subsequent recombination of mutated DNA and excision of the plasmid DNA from the bacterial cell, 6605-Δ*gacA* and 6605-Δ*gacS*/Δ*gacA* were obtained.

For complementation of the Δ*gacS* mutant, a 2965 bp fragment of *gacS* with putative promoter region was amplified by PCR with gacS-5'pro and gacS2 primers and was cloned in the pCR II-Blunt-TOPO vector (Invitrogen, Carlsbad, CA). A *Kpn*I and *Not*I fragment was inserted into the mini-Tn5 transposon vector pBSL118 (Alexeyev et al. 1995). The resulting plasmid was introduced into Δ*gacS* mutant by conjugation via *E. coli* S17-1 λpir. For complementation of the Δ*gacA* mutant, gacA1 and gacA6 primers were used to amplify a 1810 bp fragment of *gacA* with putative promoter region. This fragment was subcloned into pDSK519, a broad-host-range plasmid vector and was introduced into Δ*gacA* mutant by conjugation via *E. coli* S17-1 (Keen et al. 1988).

Extraction and detection of acylhomoserine lactones (AHLs)

To extract AHLs from *P. syringae,* the supernatants of 100 ml bacterial culture (OD_{600}=1.5) were filtered through a 0.45 μm-pore filter after centrifugation. AHLs in the filtrates were extracted from the supernatants with an equal volume of ethyl acetate, and evaporated to dryness at less than 40˚C. Residues were dissolved in 200 μl of ethyl acetate.

AHLs and extracts from the supernatants with ethyl acetate (20 μl) were spotted on C_{18} reversed-phase thin layer chromatography (TLC) plates (RP-18F 254S, Merck, Darmstadt,

Germany), then developed with methanol/water (60:40, v/v). After development, the dried TLC plate was overlaid with 50 ml of semisolid LB agar medium containing 6 ml of an overnight culture of *Chromobacterium violaceum* CV026. After overnight incubation at 30˚C, AHLs were visualized as violet spots by the induction of violacein production (McClean et al. 1997).

Swimming and swarming motility

For the swimming assay, bacteria grown on King's B (KB, King et al. 1954) agar plates were inoculated with a toothpick onto the center of a swimming plate (MMMF; 50 mM potassium phosphate, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl with 10 mM mannitol and fructose, 0.25% agar). For the swarming assay, the density of overnight culture was adjusted to an OD_{600} of 0.3 with 10 mM $MgSO_4$ and 3μl aliquots were put on the center of plate (SWM, 0.5% peptone (DIFCO, Detroit, MI, USA), 0.3% yeast extract (DIFCO), and 0.4% granulated agar (DIFCO), Kinscherf et al. 1999). The plates were then incubated for 24h at 25˚C for swimming assay and 27˚C for swarming assay.

Plant materials and bacterial inoculation

Tobacco (*Nicotiana tabacum* L. cv. Xanthi NC) and tomato (*Lycopersicon esculentum* L. cv. Moneymaker) were grown in a growth chamber at 26˚C. For the inoculation experiment by the spray method, each bacterium was suspended in 10 mM $MgSO_4$ containing 0.02% Silwet L77 (OSI Specialities, Danbury, CT) to a density of $2X10^8$ cfu/ml. To investigate the HR cell death, tomato leaves were infiltrated with a needle-less syringe at $2X10^8$ or $2X10^7$ cfu/ml, respectively. The inoculated leaves were incubated in a growth cabinet at 23˚C.

Pigment production

Ten microliter aliquots of bacterial suspension adjusted to OD_{600}=1.0 were spotted on KB agar plates and allowed to dry for 10 min. After incubation at 27˚C for overnight, pigment production was investigated under ultraviolet illumination.

RNA preparation and Northern blot analysis

P. syringae strains grown in LB medium at 28˚C to 0.3 OD_{600} were further incubated in MMMF medium for several hours; then total RNA was extracted using a High Pure RNA Isolation kit (Roche, Mannheim, Germany). Ten micrograms of total RNA was used for Northern blot hybridization. The probes used in this study were part of DNA fragments of

psyI, psyR, algT, and *hrpL*. The DNA fragments of *psyI* (1 to 680 in AB266105), *psyR* (1 to 744 in AB266106), *algT* (1 to 582 in AB266107), and *hrpL* (1 to 555 in AB266108) were amplified by PCR with their respective set of primers (Supplemental table) using genomic DNA of *P. syringae* pv. *tabaci* as the template DNA and cloned into a pGEM-T-Easy plasmid. The *hrpA* probe was amplified by PCR with T7 and SP6 primers using pNCHU665 as a template. Probes were prepared with a PCR DIG Probe Synthesis kit (Roche). Hybridization was performed in hybridization buffer (7.0% sodium dodecyl sulfate (SDS), 0.1% lauroylsarcosine, 5 X SSC, 2.0% blocking solution, and 0.05 M Na_2HPO_4 (pH 7.2)) at 65˚C for 18 hr. After hybridization, membranes were washed in 2 X SSC, 0.1% SDS at 65˚C for 20 min, then in 0.5 X SSC, 0.1% SDS at 65˚C for 30 min. Hybridized signals were detected by anti-DIG antibody conjugated with alkaline phosphatase (Roche) and its chemiluminescent substrate CDP-star (Roche).

Adhesion assay

Adhesion ability was investigated by the method described by Taguchi et al. (2006b). Bacteria cultured overnight in LB medium containing 10 mM $MgCl_2$ at 27˚C were centrifuged and suspended in MMMF medium at 0.1 OD_{600}. Aliquots (200 µl) of bacteria

were transferred to microtiter plate wells (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and incubated at 27°C for 2 days without shaking. After incubation, the wells were washed with water 3 times. Then, adhering bacteria were stained with 200 µl of 0.5% crystal violet for 45 min and washed five times with water. Crystal violet-stained cells were extracted with 200 µl of 95% ethanol and quantified by the absorbance at OD_{595}.

Preparation and detection of EPS

MMMF medium (30 ml) was inoculated with one ml of bacteria at 0.1 OD_{600} and incubated until it reached 0.3 OD_{600}. Following removal of bacteria by centrifugation, EPSs in the supernatants were precipitated by the addition of 3 volumes of 95% ethanol at -20˚C as previously described (Lopez-Garcia et al. 2001). The precipitated residues were dissolved in 200 µl of 0.5 M NaCl.

EPS was separated by tricine-SDS-polyacrylamide gel electrophoresis (PAGE) with 20% acrylamide (Bio-Rad, Hercules, CA, USA). Samples were mixed with 6 x sample buffer (125 mM Tris [pH 6.8], 48% glycerol, 0.3% bromophenol blue) with 12% SDS and 24% β-mercaptoethanol). A low-range protein molecular weight marker, Rainbow marker 756 (Amersham, Roosendaal, The Netherlands), was used as a molecular standard marker. Silver

staining was performed according to manufacturer's instructions (2D-Silverstaining Reagent II, Daiichi Pure Chemicals, Tokyo, Japan).

Results

Sequence analysis of *gacS* and *gacA* and creation of deletion mutants

The nucleotide sequences of *gacS* and *gacA* genes were determined. The predicted numbers of amino acids encoded and molecular masses were 918 amino acids and 100.2 kDa for GacS and 222 amino acids and 24.2 kDa for GacA, respectively. The deduced amino acid sequences of GacS and GacA are highly homologous to those of *P. syringae* pv. *phaseolicola* 1448A, pv. *tomato* DC3000, and pv. *syringae* B728a at more than 94% and 97%, respectively. However, these homologies to *Erwinia carotovora* subsp. *atroseptica* are only 37% for GacS and 57% for GacA. *GacS* and *gacA* deletion mutants were created by generation of an internal deletion within each open reading frame and homologous recombination with *gacS* and *gacA* genes of the wild-type bacterium. The specific mutations in *gacS* and *gacA* were confirmed by respective PCRs as shown in Fig. 1C and 1D. PCR to amplify the genomic region containing *gacS* gene resulted in amplification of a 2.3 kb DNA fragments using genomic DNA of the wild-type bacterium as a template, while PCR using Δ*gacS* and

Δ*gacS*Δ*gacA* defective mutants resulted in 2.0 kb DNA fragments (Fig.1C). Similarly, PCR to amplify the *gacA* gene with genomic DNA of wild-type bacterium resulted in amplification of a 1.1 kb DNA fragments and in 0.7 kb DNA fragments with Δ*gacA* and Δ*gacS*Δ*gacA* defective mutants (Fig.1D). These results indicate that *gacS*- and *gacA*-specific deletion mutants were successfully obtained.

GacS and GacA are involved in AHL production

The effect of *gacS*- and *gacA*-defective mutations on AHL production was examined by a bioassay using *C. violaceum* CV026. Fig. 2A shows that *P. syringae* pv. *tabaci* 6605 produced three detectable compounds: the two major signals had the same Rf values as *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL) and *N*-hexanoyl-L-HSL. The production of all AHLs by Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants was drastically reduced compared to the wild-type. Only the putative *N*-hexanoyl-L-HSL was detected in all *gac* defective mutants.

PsyI and *psyR* have been identified as genes required for the biosynthesis and function of AHLs in *P. s*yringae pv. *tabaci* 2024 (GenBank accession no. AF110468, Shaw et al. 1997). *PsyI* and *PsyR* are *luxI* and *luxR* homologs, respectively. The *luxI* homologs encode an

AHL synthase that catalyzes the synthesis of the AHL in the presence of the cellular metabolites *S*-adenosyl-methionine and a specific acylated acyl-carrier protein in most bacterial species. Northern blot analysis of *psyI* and *psyR* also revealed that the transcript levels of these genes in Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants in LB medium were lower than in the wild-type (Fig. 2B). However, when the supernatant of the wild-type culture filtrate was added to the medium of these mutants, transcription of *psyI* and *psyR* was recovered within 3 hr of incubation (Fig. 2B). The recovery of *psyI* expression was also observed when the mixture of *N*-(3-oxohexanoyl)-L-HSL and *N*-hexanoyl-L-HSL was applied to the medium of three *gac*-defective mutants, but not by application of the supernatant prepared from the Δ*gacS*Δ*gacA* mutant strain (data not shown). These results suggest that the Gac two-component system positively controls AHL production. However, AHLs and the supernatant presumably containing AHLs recovered the expression of *psyI* and *psyR*, indicating that the expression of these genes is primarily regulated by the AHLs.

Effects of GacS and GacA on motility and pigment production

To examine the effect of the Gac two-component system on motility, Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants were inoculated on a 0.25% agar MMMF medium plate for the

swimming assay and a 0.4% agar SWM plate for the swarming assay (Kinscherf and Willis 1999). The swimming motility of these mutants and complemented strains was the same as that of the wild-type (Fig. 3A), whereas the swarming motility of these mutants was completely abolished (Fig. 3B). On the other hand, the reduction of swarming motility was complemented in pBSL*gacS* and pDSK*gacA*. These results suggest that the Gac two-component system controls swarming motility.

Like other *Pseudomonas* species (Chatterjee et al. 2003), *P. syringae* pv. *tabaci* produces a diffusible yellow pigment on KB medium, as shown in Fig. 4. However, all *gac*-defective mutants lost the ability to produce the pigment. When the ethyl acetate extract of the culture filtrate of the wild-type strain or a mixture of *N*-(3-oxohexanoyl)-L-HSL and *N*-hexanoyl-L-HSL was added to the culture medium for *gac*-defective mutants, neither swarming motility nor the pigment production ability was recovered (data not shown). Each complemented strain of pBSL*gacS* and pDSK*gacA* was also restored the ability to produce the pigment (Fig. 4).

Effects of GacS and GacA on virulence and HR induction

To examine the effects of mutation of the Gac two-component system on virulence, we tested

the tobacco response to the wild-type, Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants of *P. syringae* pv. *tabaci* 6605. Tobacco leaves were sprayed with 2 x 10^8 cfu/ml of bacterial suspensions. The wild-type strain caused disease symptoms at 12 days after inoculation, whereas the Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants failed to cause any disease symptoms (Fig. 5A). On the other hand, disease symptoms were again appeared when complemented strains with pBSL*gacS* and pDSK*gacA* were used as inocula (data not shown). After the inoculation of tobacco leaves with the wild-type and Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants by the same method, bacterial populations were measured. All *gac*-defective mutants had less growth than WT strain by 7 days after spray-inoculation (Fig. 5B).

Interestingly, none of the *gac*-defective mutants lost the ability to induce HR. Infiltration with all bacterial suspensions at both 2 X 10^7 and 2 X 10^8 cfu/ml induced HR in nonhost tomato leaves (Fig. 5C).

GacS and GacA regulates adhesion

Because it is known that swarming motility is linked to the capacity for biofilm formation, accumulation of EPS, and virulence in several animal pathogens (Kirov et al. 2002), we investigated the effect of the mutation in *gac* genes on the adhesion ability by measuring the

amount of bacterial cells adhering to microtiter plates as described by Taguchi et al. (2006b). The adhesion ability of Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants was remarkably reduced compared to that of the wild-type (Fig. 6A), indicating that the Gac two-component system is required for adhesion ability. We investigated the expression of *algT,* a gene encoding the alternate sigma factor σ^{22}, a transcription factor in alginate synthesis (Keith and Bender 1999) and EPS accumulation. The expression of *algT* was detected in the wild-type but not in the Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants (Fig. 6B). Similar to the swarming motility, *algT* expression was not recovered even in the presence of the supernatant from the culture medium of the wild-type strain. Accumulation of EPS was strongly detected as 6.5 kDa band by tricine-SDS PAGE of the wild-type strain but only weakly from Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants (Fig. 6C).

Involvement of GacS and GacA on *hrp* gene expression

It is well known that a type III secretion system is essential for virulence in many phytopathogenic bacteria, including *P. syringae* pv. *tabaci* (Marutani et al. 2005). Because the virulence of the Δ*gacS*, Δ*gacA*, and Δ*gacS*/Δ*gacA* mutants in *P. syringae* pv. *tabaci* 6605 was remarkably reduced, we investigated the gene expression of *hrpL* and *hrpA*, which

encode an alternate sigma factor and Hrp pili, respectively. Expression of *hrpL* and *hrpA* genes in the wild-type strain was induced by incubation in the nutrient-poor MMMF medium within 1 h (Fig. 7A). However, the induction of *hrpL* was almost completely abolished and that of *hrpA* was remarkably reduced in the Δ*gacS*, Δ*gacA*, and Δ*gacS*Δ*gacA* mutants (Fig. 7A). To examine the potential effects of extracellular compounds of the wild-type strain on the induction of *hrp* genes, we applied the supernatant from the wild-type culture filtrate to the MMMF medium of each *gac*-defective mutant strains. However, the expression of *hrpL* and *hrpA* genes was not affected by this exogenous application (Fig. 7B). These results indicate that *hrp* gene expression is regulated by the Gac two-component system and is independent of the quorum sensing system.

Discussion

In this paper, we report the effects of the mutation of *gac* genes in *P. syringae* pv. *tabaci* 6605. The results showed the Gac two-component system controls quorum sensing, swarming ability, production of pigment, EPS production, adhesion and *hrp* gene expression, and is altogether indispensable for virulence. Thus there are complicated regulatory network under Gac two components regulatory system. And some phenotypes that are impaired in *gac*

mutants are not necessarily regulated directly by Gac two-component system.

In bacteria, quorum sensing is a system for bacterium-bacterium communication via small signal molecules, so-called quorum-sensing molecules or autoinducers. The major quorum-sensing molecules produced in *P. syringae* pv. *tabaci* 6605 are *N*-hexanoyl-L-homoserine lactone and *N*-(3-oxohexanoyl)-L-homoserine lactone. Further, the mutants of this pathogen that had lost the ability to produce quorum-sensing molecules had remarkably reduced virulence on host tobacco (Taguchi et al. 2006a).

Because it was reported that the Gac two-component system is important for the production of AHLs in *P. syringae* pv. *syringae* B728a (Kinscherf and Willis 1999, Kitten et al., 1998) and in *P. aureofaciens* (Chancey et al. 1999), we investigated the correlation between the Gac two-component system and production of AHLs, as well as virulence factors including *hrp* gene expression and flagellar motility. All *gac*-defective mutants of this pathogen had a remarkably reduced ability to produce AHLs (Fig. 2A). The application of the AHL-containing supernatant from bacterial cultures of the wild-type or AHLs to the medium of *gac*-defective mutants restored the expression of the *psyI* and *psyR* genes (Fig. 2B). This suggests that the *psyI* and *psyR* genes are autoregulated by AHLs when Gac is absent. Thus, the Gac two-component system is required for the production of AHLs and expression of *psyI*

and *psyR* in *P. syringae* pv. *tabaci* 6605.

Although swimming motility was not affected by the *gac*-defective mutation, swarming motility was abolished in these mutants. Further, exogenous application of AHLs did not recover swarming motility. AHLs are also reported to have no effect on swarming motility in *P. syringae* pv. *syringae* B728a (Kinscherf and Willis 1999). These results indicate that the Gac two-component system regulates swarming motility independent of AHLs.

The ability to produce pigment was also impaired in *gac*-defective mutants (Fig. 4). Further, loss of pigment production in the mutants was not recovered by the application of AHLs. *P. syringae* pv. *syringae* strains belong to the fluorescent *Pseudomonas* group, which generally produces Fe(III)-chelating siderophores, pyoverdins, that fluorescence under UV light (Bultreys et al. 2003). It has reported that siderophore biosynthesis is controlled by a quorum-sensing system in *P. aeruginosa* (Stintzi et al. 1998), suggesting that siderophore production might be regulated by the Gac two-component system in *P. syringae* pv. *tabaci* 6605.

Adhesion ability requires biofilm formation, and EPS production and *algT* expression contribute to biofilm formation. Production of alginate, one of the major EPSs was reduced in

the *gac*-defective mutants (Fig. 6C), indicating alginate production is controlled by the Gac two-component system, as in *Azotobacter vinelandii* (Castaneda et al. 2001) and *P. syringae* pv. *syringae* B728a (Willis et al. 2001). The *algT* transcript level was also reduced in *gac*-defective mutants (Fig. 6B). However, no effect of the *gacA* mutation on *algT* and *algD* was observed in *P. syringae* pv. *tomato* DC3000 (Chatterjee et al., 2003). This indicates that the Gac two-component system works differently in different pathovars. The reduced expression of the *algT* gene in gac-defective mutants was not recovered by the exogenous application of AHLs. This suggests that alginate production is also independent of quorum sensing system.

Previously the Hrp TTSS and flagellar motility were shown to be required for full virulence of *P. syringae* pv. *tabaci* 6605 in host tobacco plants (Marutani *et al*., 2005). In this study, we revealed that the Gac two-component system is required not only for production of AHLs (Fig. 2), pigment (Fig. 4) and EPS (Fig. 6), but also flagellar swarming motility (Fig. 3) and *hrp* gene expression (Fig. 7). Although the importance of the Gac two-component system in *hrp* gene expression and flagellar motility was also reported in *P. syringae* pv. *tomato* DC3000 (Chatterjee et al., 2003), the regulatory hierarchy in the expression of virulence is not well known. The impaired expression of *hrp* genes was not recovered by the

exogenous application of AHL in *gac*-defective mutants (Fig. 7). The result indicates that *hrp* gene expression was not regulated by the quorum sensing system.

Unlike *P. syringae* pv. *tomato* DC3000 (Chatterjee et al., 2003), the *gac*-defective mutants of *P. syringae* pv. *tabaci* 6605 retained the ability to induce HR on nonhost leaves. We previously found that a *hrp*-defective mutant of *P. syringae* pv. *tabaci* 6605 retained the ability to induce HR in nonhost tomato leaves (Marutani et al. 2005). Here we showed the Gac two-component system controls the expression of *hrpL* and *hrpA* but not flagellar swimming motility. Because flagellin is a major elicitor of HR in *P. syringae* pv. *tabaci* 6605 (Marutani et al., 2005), *gac*-defective mutants of this pathogen retained the ability to induce HR in nonhost tomato leaves.

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Table 1 Bacterial strains and plasmids

Bacterial strain/plasmid	Relevant characteristics	Reference or source
Escherichia coli		
DH5α	*F⁻ λ⁻ø80dLacZ ΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17($r_K^-m_K^+$) supE44 thi-1 gyrA relA1*	Takara, Kyoto, Japan
S17-1	*thi pro hsdR hsdR hsdM⁺ recA(chr::RP4-2-Tc::Mu-Km::Tn7)*	Schaefer et al. (1994)
P. syringae pv. *tabaci*		
Isolate 6605	wild-type isolated from tobacco, Nal^r	Ishiga et al. (2005)
6605-Δ*gacS*	Isolate 6605 Δ*gacS*, Nal^r	This study
6605-Δ*gacA*	Isolate 6605 Δ*gacA*, Nal^r	This study
6605-Δ*gacS*Δ*gacA*	Isolate 6605 Δ*gacS*Δ*gacA*, Nal^r	This study
6605-Δ*gacS* (pBSL-*gacS*)	Isolate 6605 Δ*gacS*, Nal^r, (pBSL-*gacS*)	This study
6605-Δ*gacA* (pDSK-*gacA*)	Isolate 6605 Δ*gacA*, Nal^r, (pDSK-*gacA*)	This study
Plasmids		
pGEM-TEasy	Cloning vector, Amp^r	Promega, Tokyo, Japan
pK18*mob*SacB	Small mobilizable vector, Km^r, sucrose sensitive (*sac*B)	Invitrogen
pGEMgacS	2,757 bp *gacS* subcloned into pGEM-TEasy	This study
pGEMgacS2	2,320 bp truncated *gacS* subcloned into pGEM-TEasy	This study
pGEMgacA1	2,227 bp *gacA* subcloned into pGEM-TEasy	This study
pGEMgacA2	792 bp *gacA* subcloned into pGEM-TEasy	This study
pGEMhrpL	555 bp *hrpL* subcloned into pGEM-TEasy	This study
pGEMpsyI	681 bp *psyI* subcloned into pGEM-TEasy	This study
pGEMpsyR	744 bp *psyR* subcloned into pGEM-TEasy	This study
pGEMalgT	582 bp *algT* subcloned into pGEM-TEasy	This study
pNCHU665	*hrpA* subcloned into pGEM-TEasy	H.C. Huang (unpublished)
pMgacS	Truncated *gacS* subcloned into pK18*mob*SacB	This study
pMgacA	Deleted *gacA* fragments subcloned into pK18*mob*SacB	This study
pBSL118	Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp^r, Km^r	Alexeyev et al. (1995)
pBSL-*gacS*	*gacS* containing 2965 bp DNA fragment in pBSL118	This study
pDSK519	Broad-host-range cloning vector, Km^r	Keen et al. (1988)
pDSK-*gacA*	*gacA*-containing 1810 bp DNA fragment in pDSK519	This study

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

Figure legends

Fig. 1. Generation of Δ*gacS*- (**A**) and Δ*gacA*- (**B**) defective mutants. The details are described in Materials and Methods. Confirmation of specific deletion of *gacS* (**C**) and *gacA* (**D**) genes. Two sets of primers (gacS1 and gacS3 for *gacS*, gacA5 and gacA6 for *gacA*) were used to amplify DNA fragments by genomic PCR to confirm *gacS*- and *gacA*-specific deletion. The approximately 2.3 kb DNA bands indicate an intact *gacS* gene, whereas 2.0 kb DNA bands indicate a *gacS*-specific deletion in **C**. Similarly, 1.1 kb DNA bands indicate an intact *gacA* gene, whereas 0.7 kb DNA bands indicate a *gacA*-specific deletion in **D**. WT, wild-type; ΔS, Δ*gacS* mutant; ΔA, Δ*gacA* mutant; and ΔSA, Δ*gacS*/Δ*gacA* double mutant.

Fig. 2. Detection of *N*-acyl-L-homoserine lactones (AHLs) and gene expression for *psyI* and *psyR*. **A**, Detection of AHLs on TLC plate using *C. violaceum* CV026. The ethyl acetate extract from 10 ml of culture filtrate was spotted on the TLC plate. As standards for AHLs, synthetic *N*-(3-oxohexanoyl)-L-homoserine lactone (o-C6) and *N*-(3-hexanoyl)-L-homoserine lactone (C6) were also spotted on the same TLC plate. **B**, Expression of *psyI* and *psyR* mRNA using Northern blot analysis. Bacteria were grown overnight in LB medium supplemented with 10 mM $MgCl_2$. Two bacterial suspensions were prepared after adjusting the bacterial concentration to 1.5 OD_{600} with the same medium. One suspension was further incubated for 3 h, whereas the medium of the other was replaced with the supernatant of

overnight culture of the wild-type strain after filter sterilization. The quality and quantity of RNA are shown as rRNA staining by ethidium bromide. Bacterial strains are described in the legend of Fig. 1. o-C6, *N*-(3-oxohexanoyl)-L-HSL; C6, *N*-hexanoyl-L-HSL.

Fig. 3. Bacterial motility. **A,** Swimming motility was examined in 0.25% agar MMMF medium. Bacteria were inoculated into the center of a plate with a toothpick and incubated for 24h at 23˚C. **B,** Swarming motility was examined in 0.4% agar SWM medium. Cell density was adjusted to an OD_{600} of 0.3 with 10 mM $MgSO_4$ and 3 µl aliquots were inoculated and incubated for 24 h at 27˚C. ΔS-C, complemented strain of Δ*gacS* mutant with pBSL-*gacS*; ΔA-C, complemented strain of Δ*gacA* mutant with pBSL-*gacA*. Other bacterial strains are described in the legend of Fig. 1.

Fig. 4. Pigment production. Ten microlittle aliquots of *P. syringae* pv. *tabaci* adjusted to an OD_{600} of 1.0 were spotted on KB medium, and incubated for 24 h at 27˚C. **A,** Colony observation in the light field. **B,** Pigment production was examined under ultraviolet illumination. Bacterial strains are described in the legends of Fig. 1 and Fig. 3.

Fig. 5. Inoculation tests on host tobacco and nonhost tomato leaves. **A,** Tobacco leaves were inoculated by spray-methods using bacterial suspensions with a cell density of 2×10^8 cfu/ml. Photographs were taken 12 days after inoculation. **B,** Bacterial growth of different strains of

P. syringae pv. *tabaci* 6605 in tobacco leaves. Each bacterial strain was spray inoculated on tobacco leaves at a cell density of 2 X 10^8 cfu/ml. Bacterial populations were measured 1, 3, and 7 days after inoculation. **C,** The bacterial suspensions with a density of 2 X 10^7 and 2 X 10^8 cfu/ml were infiltrated into tomato leaves to detect HR. Photographs were taken 2 days after inoculation. Bacterial strains are described in the legend of Fig. 1.

Fig. 6. Adhesion ability, *algT* gene expression, and EPS production of *P. syringae* pv. *tabaci.* **A,** Adhesion ability was evaluated as absorbance at OD_{595} after staining bacterial cells with crystal violet. **B,** Expression of *algT* was investigated by Northern blot hybridization as described in the legend of Fig. 2B. The quality and quantity of RNA are shown as staining of rRNA by ethidium bromide. **C,** EPS was detected by silver staining. Bacterial strains are described in the legend of Fig. 1.

Fig. 7. Effect of *gacS*- and *gacA*-defective mutations on *hrp* gene expression. **A**, Bacterial strains grown in LB medium supplemented with 10 mM $MgCl_2$ were suspended in MMMF medium at 0.3 OD_{600}. Then total RNA was purified after 0, 1, and 2 h incubation, and *hrpL* and *hrpA* gene expression was analyzed. **B**, Effects of extracellular compounds of the wild-type strain of *P. syringae* pv. *tabaci* 6605 on *hrp* gene expression in *gac*-defective mutant strains. The supernatant of the culture filtrate of wild-type strain cultured overnight

was exogenously applied to MMMF medium of each bacterium, then expression of *hrp* genes was examined. Experimental conditions are described in the legend of Fig. 2B. Bacterial strains are described in the legend of Fig. 1.

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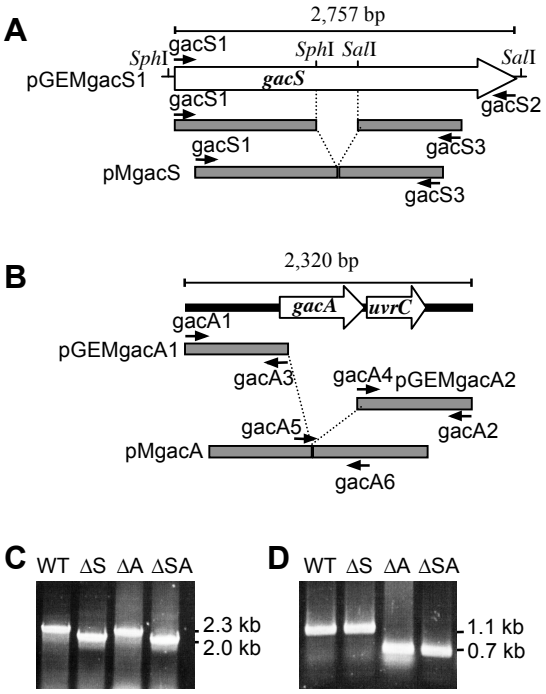
Fig. 1



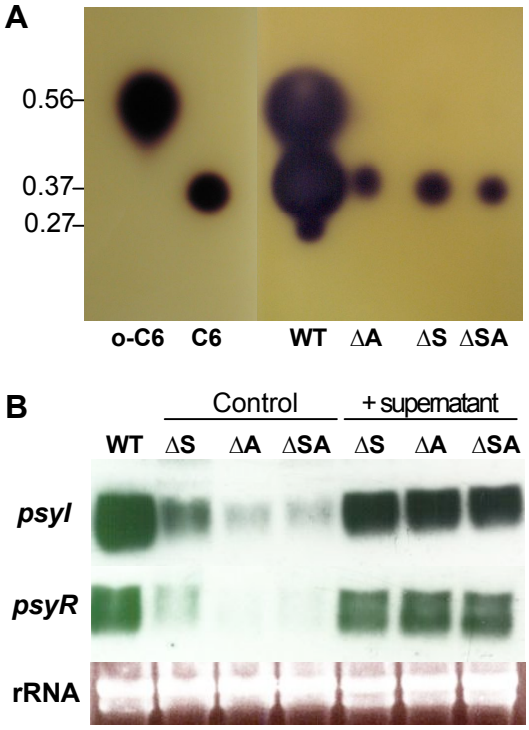

A
0.56
0.37
0.27
o-C6
C6
WT
ΔA
ΔS
ΔSA
B
Control
+ supernatant
WT
ΔS
ΔA
ΔSA
ΔS
ΔA
ΔSA
psyI
psyR
rRNA

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Fig. 3

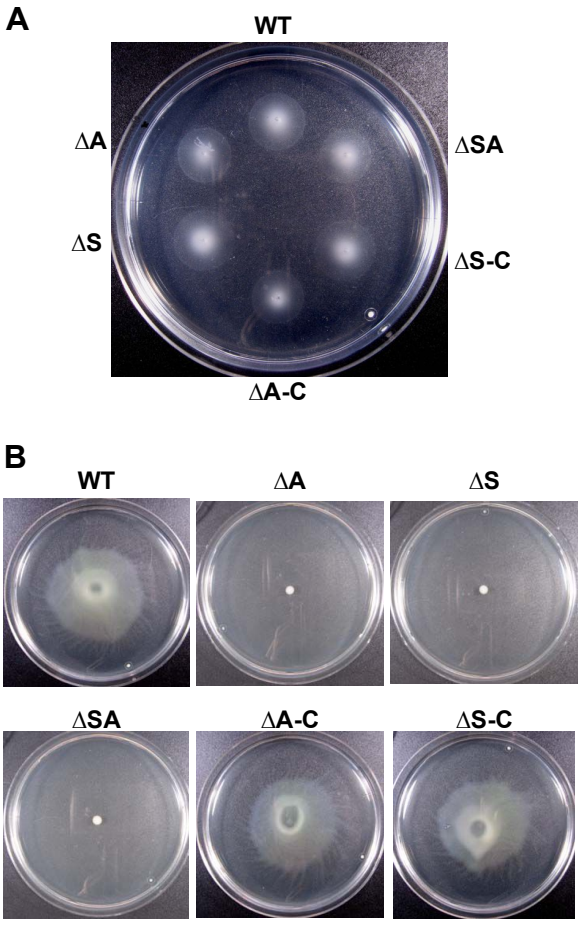

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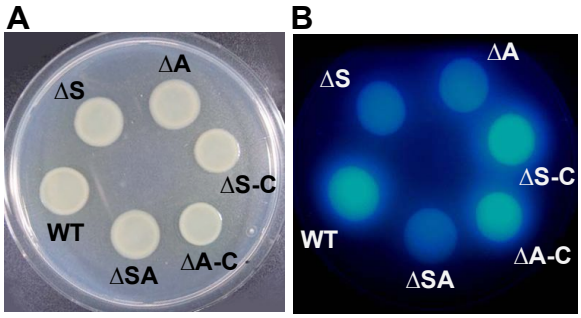
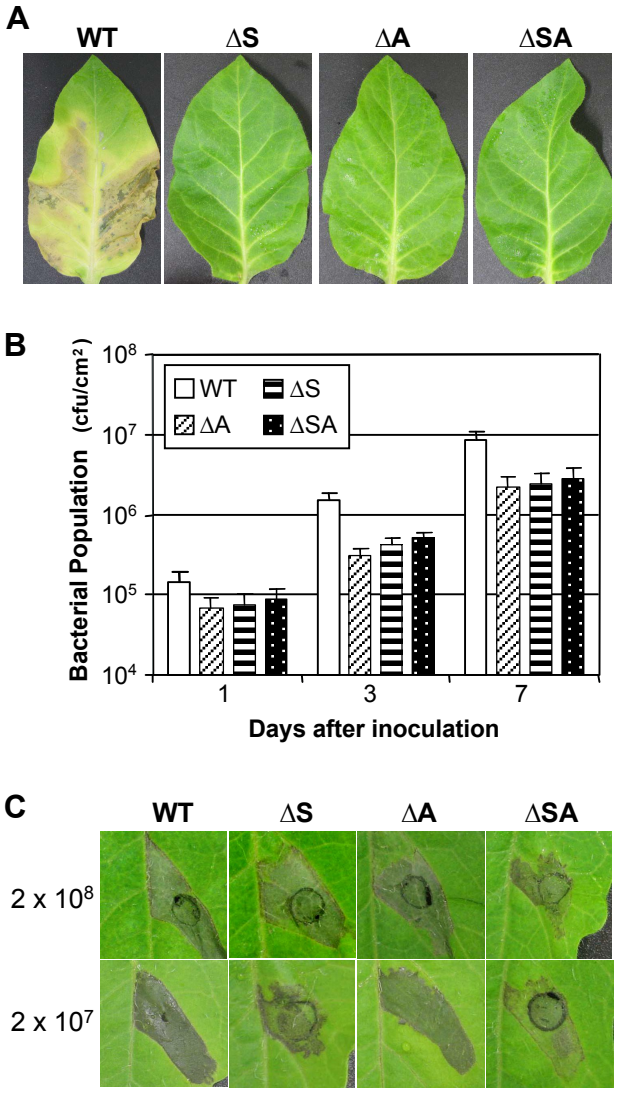
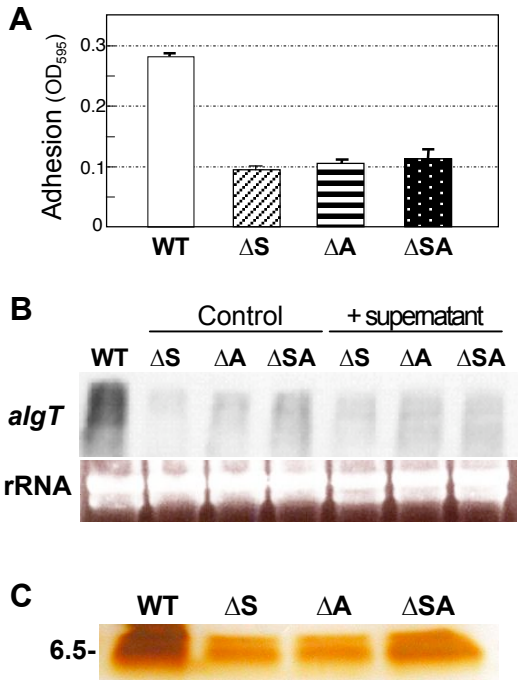
Fig. 4




A
WT
ΔS
ΔA
ΔSA
B
Bacterial Population (cfu/cm²)
10⁸
10⁷
10⁶
10⁵
10⁴
WT
ΔS
ΔA
ΔSA
1
3
7
Days after inoculation
C
WT
ΔS
ΔA
ΔSA
2 x 10⁸
2 x 10⁷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Adhesion (OD_{595})
0.3
0.2
0.1
0
WT
ΔS
ΔA
ΔSA
B
Control
+ supernatant
WT
ΔS
ΔA
ΔSA
ΔS
ΔA
ΔSA
algT
rRNA
C
WT
ΔS
ΔA
ΔSA
6.5-

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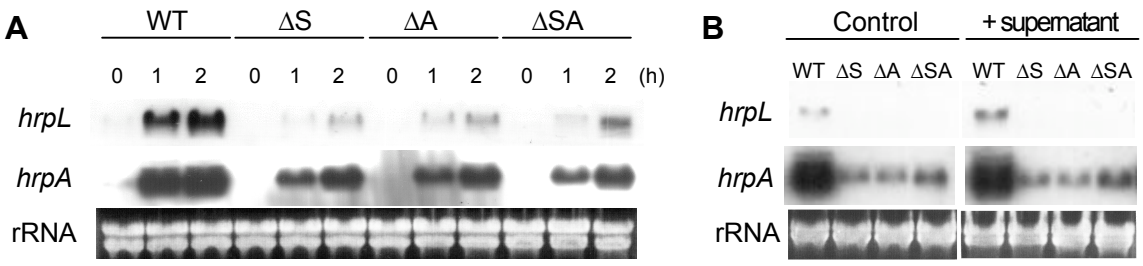
Fig. 7



Supplemental table Primer sequences

primer name	Sequence (5'-3')
gacS1	GTGCTGACCAAGCTGGGTA
gacS2	CCATTACGCTGTCACCCGCG
gacS3	CGGGCTTGGTCAGGTAGTCGTC
gacS-5'pro	AACGGCAAACGGCCAAAGCC
gacA1	CCTTTCTTGCGCCCTGCCAG
gacA2	GTTCTTGCTGCCCAGCACCC
gacA3	CGGGATCCGCCTGTCCGAACAAGGTGAT
gacA4	CGGGATCCTGGCTTTGCTCGCTGTACGT
gacA5	TTGATTAAGGTGCTAGTTGTCGAT
gacA6	CTTCTGCAGCAGGCTGAGGC
hrpL5	ATGTTTCCGAACCTAGTGATCCTT
hrpL3	TCAGGCGAACGGGTCAATCTGCTG
PsyI5	ATGTCGAGCGGGTTTGAGTTTCAG
PsyI3	TCAGGCAGCGCTGTGCATGCAGGC
PsyR5	ATGGAGGTTCGTACCGTGAAAGCC
PsyR3	TCAGACCATGCCCATGTTGATGGC
AlgT5	ATGCTAACCCAGGAAGAGGA
AlgT3	TCAGGATTCCTGCAACAACG