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Dynamic Regulation of GacA in Type III Secretion, Pectinase Gene Expression, Pellicle Formation, and Pathogenicity of *Dickeya dadantii* (*Erwinia chrysanthemi* 3937)

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***Dickeya dadantii* (*Erwinia chrysanthemi* 3937) secretes exoenzymes, including pectin-degrading enzymes, leading to the loss of structural integrity of plant cell walls. A type III secretion system (T3SS) is essential for full virulence of this bacterium within plant hosts. The GacS/GacA two-component signal transduction system participates in important biological roles in several gram-negative bacteria. In this study, a *gacA* deletion mutant (Ech137) of *D. dadantii* was constructed to investigate the effect of this mutation on pathogenesis and other phenotypes. Compared with wild-type *D. dadantii*, Ech137 had a delayed biofilm-pellicle formation. The production of pectate lyase (Pel), protease, and cellulase was diminished in Ech137 compared with the wild-type cells. Reduced transcription of two endo-Pel genes, *pelD* and *pelL*, was found in Ech137 using a green fluorescence protein-based fluorescence-activated cell sorter promoter activity assay. In addition, the transcription of T3SS genes *dspE* (an effector), *hrpA* (a structural protein of the T3SS pilus), and *hrpN* (a T3SS harpin) was reduced in Ech137. A lower amount of *rsmB* regulatory RNA was found in *gacA* mutant Ech137 compared with the wild-type bacterium by quantitative reverse-transcription polymerase chain reaction. Compared with wild-type *D. dadantii*, a lower amount of *hrpL* mRNA was observed in Ech137 at 12 h grown in medium. Although the role of RsmA, *rsmB*, and RsmC in *D. dadantii* is not clear, from the regulatory pathway revealed in *E. carotovora*, the lower expression of *dspE*, *hrpA*, and *hrpN* in Ech137 may be due to a post-transcriptional regulation of *hrpL* through the Gac-Rsm regulatory pathway. Consequently, the reduced exoenzyme production and Pel gene expression in the mutant may be partially due to the regulatory role of *rsmB*-RsmA on exoenzyme expression. Similar to in vitro results, a lower expression of T3SS and pectinase genes of Ech137 also was observed in bacterial cells inoculated into *Saintpaulia ionantha* leaves, perhaps accounting for the observed reduction in local maceration. Interestingly, compared with the wild-type *D. dadantii*, although a lower concentration of Ech137 was observed at day 3 and 4 postinoculation, there is no significant difference in bacterial concentration between the wild-type bacterium and Ech137 in the early stage of infection. Finally, the nearly abolished systemic invasion ability of Ech137 suggests that GacA of *D. dadantii* is essential for the pathogenicity and systemic movement of the bacterium in *S. ionantha*.**

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Two-component signal transduction systems (TCSTS) are widely distributed in bacteria (Azcarate-Peril et al. 2005; Dalton et al. 2006; Merighi et al. 2006; Venkatesh et al. 2006). These regulatory systems are used by organisms to respond to environmental stimuli and adapt to different environmental conditions. In enteric bacteria, the GacS/GacA TCSTS has been reported to play important roles in various biological functions (Chatterjee et al. 2003; Cui et al. 2001; Heeb et al. 2005; Reimmann et al. 2005). GacS is the putative sensor kinase and GacA is the response regulator. Although the signal molecule for GacS autophosphorylation is still unknown, GacS is suggested to activate GacA and the activated GacA works as a transcriptional activator and further activates targeted genes. In animal pathogens such as *Salmonella typhimurium*, the GacS/GacA homolog, BarA/Sir, is responsible for HilA-dependent regulation of pathogenicity island SPII (Heeb and Haas 2001). In plant-beneficial bacteria, the GacS/GacA homolog was shown to be essential for expression of biocontrol factors for disease control (Chancey et al. 2002).

In phytopathogens such as *Pseudomonas syringae* and *Erwinia carotovora*, the regulatory roles of GacS/GacA have been suggested to channel through the regulator of secondary metabolism (Rsm) system (Chatterjee et al. 2003; Cui et al. 2001). Rsm is a novel type of post-transcriptional regulatory system that plays a critical role in gene expression. RsmA, *rsmB*, and RsmC are the major components of this global regulatory system. RsmA is a small RNA-binding protein that acts by lowering the half-life of the mRNA species (Cui et al. 1995). *rsmB* is an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect by forming an inactive ribonucleoprotein complex (Cui et al. 1995; Liu et al. 1998). RsmC controls the production of RsmA and *rsmB* RNA by positively regulating *rsmA* and negatively controlling *rsmB* (Cui et al. 1999).

In plant pathogens, GacS/GacA TCSTS has been studied intensively. Many virulence factors, including pectate lyase (Pel), exoprotease, tabtoxin, and syringomycin production, are found

to be regulated by GacS/GacA homologues in phytopathogens (Chatterjee et al. 2002, 2003). In *P. syringae* pv. *tomato*, GacA acted as a master regulator of controlling regulatory RNA, several transcriptional activators, and alternative sigma factors (Chatterjee et al. 2003). In *E. carotovora* subsp. *carotovora*, GacA stimulated transcription of a Pel gene (*pel-1*), a polygalacturonase gene (*peh-1*), and a cellulase gene (*celV*) (Cui et al. 2001; Hyytiainen et al. 2001). A low level of *rsmB* and *hrpN* transcript also was observed in *gacA* or *gacS* mutants of *E. carotovora* subsp. *carotovora* (Cui et al. 2001). In *E. carotovora*, RsmA is suggested to lower the stability of the mRNA of *pel-1*, *peh-1*, *celV*, and *hrpN* transcripts and reduce the production of Pels, polygalacturonases, cellulases (Cels), and Harpin proteins (Cui et al. 2001).

Dickeya dadantii 3937 (Ech3937) secretes proteases (Prt) through the type I secretion system (Dahler et al. 1990; Delepelaire and Wandersman 1989). The bacterium also secretes an array of Cel and Pel through the type II secretion system (T2SS) (Andro et al. 1984; Bortoli-German et al. 1995; Condemine et al. 1992; Hugouvieux-Cotte-Pattat et al. 1996). The Ech3937 genome-sequencing project revealed that this bacterium encodes a complete type III secretion system (T3SS) (*unpublished results*), and a T3SS is important for the full virulence of Ech3937 (Bauer et al. 1994, 1995; Yang et al. 2002, 2007). Moreover, recent work of Yap and associates revealed that the T3SS of Ech3937 that was involved in biofilm and pellicle formation and a T3SS regulatory pathway was elucidated by observing pellicle formation in T3SS regulatory gene mutants (Yap et al. 2005).

Although both *D. dadantii* and *E. carotovora* subsp. *carotovora* macerate plant cell walls and cause soft rot disease, there are significant differences between these two bacteria regarding the pectinase repertoires and regulation network. In *E. carotovora*, ExpR activates *rsmA* transcription. However, the function of ExpR on *rsmA* is inhibited by N-acyl homoserine lactone (AHL) (Andersson et al. 2000; Cui et al. 2005, 2006). *E. carotovora* possesses two copies of the response regulator in the quorum sensing (QS) system, *expR1* and *expR2*. Both alleles activate *rsmA* transcription through a direct and specific interaction between ExpR and the *rsmA* promoter, but ExpR does not bind *ahlI* (*expI* homolog), *pel-1*, or *rsmB*, nor does ExpR activate expression of these genes (Andersson et al. 2000; Cui et al. 2005). By contrast, ExpR protein of *D. dadantii* binds specifically to the promoter regions of *expI*, *expR*, and five major *pel* genes (Castang et al. 2006; Nasser et al. 1998). However, mutation of *expI*, the gene for QS signal synthase, has little effect on the expression of *hrpN* (a T3SS harpin), the production of pectinase, and the virulence of *D. dadantii* (Ham et al. 2004). Although the expression of exoenzymes and T3SS in *gacA* mutant of *E. carotovora* subsp. *carotovora* in medium culture was reduced, it is unclear how GacA regulates the expression of these genes in planta and affects pathogenicity (Chatterjee et al. 2002; Cui et al. 2001). To investigate whether GacA activates the transcription of *rsmB* and whether the Gac-Rsm global regulatory pathway of *D. dadantii* has conserved functions on exoenzyme production, T3SS gene expression, and pathogenesis, in this study, we used quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and a green fluorescence protein (GFP)-based fluorescence-activated cell sorter (FACS) promoter activity assay to compare the expression patterns of T3SS and pectinase genes in wild-type Ech3937 and its *gacA* mutant, Ech137. The consequence of the *gacA* mutation on the bacterial biofilm-pellicle formation and exoenzyme production also was examined. Finally, the effect of GacA on virulence gene expression, bacterial concentration, local maceration, and systemic invasion ability of Ech3937 in planta was examined.

Table 1. Bacterial strains and plasmids

Strains, plasmids	Characteristics[a]	Reference or source
Strains		
Escherichia coli S17-1 λ-pir	λ-pir Lysogen of S17-1	V. de Lorenzo, Madrid
Ech3937	Wild-type strain isolated from *Saintpaulia ionantha*	D. Expert, Paris
Ech-Rif	Ech3937 rifampicin resistant random mutant	This work
Ech137	Δ*gacA*::Kan, Km^r, Ech-Rif derivative	This work
Ech-Rif (pDspE)	Ech3937-Rif containing plasmid pDspE	This work
Ech137(pDspE)	Ech137 containing plasmid pDspE	This work
Ech-Rif (pHrpA)	Ech3937-Rif containing plasmid pHrpA	This work
Ech137(pHrpA)	Ech137 containing plasmid pHrpA	This work
Ech-Rif (pHrpN)	Ech3937-Rif containing plasmid pHrpN	This work
Ech137(pHrpN)	Ech137 containing plasmid pHrpN	This work
Ech-Rif (pHrpL)	Ech3937-Rif containing plasmid pHrpL	This work
Ech137(pHrpL)	Ech137 containing plasmid pHrpL	This work
Ech-Rif (pPelD)	Ech3937-Rif containing plasmid pPelD	This work
Ech137(pPelD)	Ech137 containing plasmid pPelD	This work
Ech-Rif (pPelL)	Ech3937-Rif containing plasmid pPelL	This work
Ech137(pPelL)	Ech137 containing plasmid pPelL	This work
Ech137 (pCLgacA)	Ech137 containing pCLgacA	This work
Plasmids		
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	Metcalf et al. 1995
pCR2.1-TOPO	PCR cloning vector, Ap^r, Km^r	Invitrogen, Carlsbad, CA, U.S.A.
pPROBE-AT	GFP promoter-probe vector, Ap^r	Miller et al. 2000
pCL1920	Low copy number plasmid	Lerner and Inouye 1990
pDspE	pProbe-AT derivative with PCR fragment containing *dspE* promoter region, Ap^r	Yang et al. 2004
pHrpA	pProbe-AT derivative with PCR fragment containing 412-bp *hrpA* promoter region, Ap^r	*Unpublished data*
pHrpN	pProbe-AT derivative with PCR fragment containing 396-bp *hrpN* promoter region, Ap^r	*Unpublished data*
pHrpL	pProbe-AT derivative with PCR fragment containing *hrpL* promoter region, Ap^r	*Unpublished data*
pPelD	pProbe-AT derivative with PCR fragment containing *pelD* promoter region, Ap^r	Peng et al. 2006
pPelL	pProbe-AT derivative with PCR fragment containing 609-bp *pelL* promoter region, Ap^r	This work
pCLgacA	pCL1920 with a 1,548-bp PCR product containing full length of *gacA*, Sp^r	This work

[a] Ap^r, Km^r, Rif^r, and Sp^r, ampicillin, kanamycin, rifampicin, and spectinomycin resistance, respectively; PCR, polymerase chain reaction; and GFP, green fluorescent protein.

RESULTS

GacA affects biofilm-pellicle formation.

Ech3937 is capable of forming a biofilm and pellicle in SOBG broth (Yap et al. 2005). A spontaneous rifampicin-resistant derivative of Ech3937, Ech-Rif, was used as a wild-type in this study (Table 1). A *gacA* deletion mutant Ech137 of Ech-Rif was constructed and confirmed by DNA sequencing analysis. The *gacA* gene of Ech137 was deleted with only 20 bp of the *gacA* open reading frame remaining. No significant difference in growth between Ech-Rif and the *gacA* mutant Ech137 was observed in M9 minimal medium (MM) (data not shown). Ech-Rif formed biofilm-pellicle in SOBG broth grown in 2 days at 28°C. However, no visible biofilm-pellicle was observed in Ech137 until grown for 3 days in SOBG broth. This delayed biofilm-pellicle formation phenotype of the mutant could be restored nearly to the wild-type level when Ech137 was complemented with a low-copy-number plasmid pCLgacA (Fig. 1A). The pellicles of Ech-Rif and Ech137 were sectioned using an ultramicrotome and the interior textures of the pellicles of the bacteria were compared using a scanning electron microscope. A more compact texture in 10-day-old pellicles was observed in Ech-Rif in comparison with Ech137 (Fig. 1B). Pellicles of both Ech-Rif and Ech137 treated with cellulase disintegrated (data not shown), suggesting that the major component, cellulose, was not altered in Ech137.

GacA regulates exoenzyme production.

Exoenzyme production, including Pel, Cel, and Prt, of the Ech-Rif and Ech137 strains was examined with semi-quantitative plate assays. Compared with Ech-Rif, reduced Pel, Cel, and Prt production was observed in Ech137 grown in MM and MM supplemented with 1% polygalacturonate (PGA) (MMP) at 36 h (Fig. 2). A spectrophotomeric assay was used to quantify Pel activity in the bacterial strains. Consistent with the results from the plate assay, lower Pel production by Ech137 was observed by the spectrophotomeric assay when the bacterial cells were grown at 36 h (late stationary phase) (Fig. 3). Compared with the Ech-Rif, a lower Pel production of Ech137 also was observed by the spectrophotomeric assay when the bacterial cells were grown at exponential phase (12 h) and beginning of stationary phase (24 h) (data not shown). The Pel, Cel, and Prt production of Ech137 was restored nearly to the wild-type bacterium level by introducing the plasmid pCLgacA containing wild-type *gacA* gene into the mutant (Figs. 2 and 3).

GacA regulates the expression of *pel* and T3SS genes.

PelD and PelL of *D. dadantii* encode endo-Pels (Lojkowska et al. 1995). PelD has higher activity on nonmethylated pectins and PelL prefers partially methylated pectins (Robert-Baudouy et al. 2000). The promoter regions of *pelD* and *pelL*, respectively, were cloned into pPROBE-AT to produce pPelD and pPelL (Table 1). The Ech-Rif and Ech137 cells carrying these GFP promoter plasmids were grown in MM-supplemented 1% PGA and the fluorescence intensity of the bacterial cells was measured with an FACS. The fluorescence intensity collected by FACS was analyzed in a four-decade log scale using CellQuest Pro software from Becton Dickinson, and the gene expression profiles were analyzed as i) total, the average GFP fluorescence intensity of total bacterial cells; ii) GFP^+ mean, average GFP fluorescence intensity of GFP expressing bacterial cells; and iii) GFP^+%, the percentage of GFP-expressing bacterial cells of the total bacterial cells (Peng et al. 2006).

To study the influence of GacA on the transcription of *pel* genes in Ech3937, the promoter activities of *pelD* and *pelL* of Ech-Rif and Ech137 were examined. Compared with Ech-Rif, a considerably lower expression of *pelD* and *pelL* was observed in *gacA* mutant Ech137 at 12 and 24 h of growth in the medium, indicating that GacA upregulated the expression of *pelD* and *pelL* (Fig. 4). The *pelD* expression in wild-type Ech-Rif is more than twofold higher than that of the *gacA* mutant Ech137 at 12 h postgrown (Fig. 4). The Ech-Rif cells carrying pPelD at 12 and 24 h were expressed at a mean fluorescence intensity (MFI) of 190 ± 17 and 459 ± 73, while the Ech137 cells carrying pPelD at 12 and 24 h were expressed with an MFI of 52 ± 3 and 324 ± 11. Similarly, the *pelL* promoter activity in the Ech-Rif cells was approximately 50% greater than that in the *gacA* mutant Ech137 cells. The MFI values of Ech-Rif (pPelL) were 31 ± 0.1 and 28 ± 0 at 12 and 24 h postgrown, respectively (Fig. 4). The MFI values of Ech137 (pPelL) were 19 ± 1 and 22 ± 1 at 12 and 24 h (Fig. 4).

Regulation of the T3SS genes by GacA has been reported in *E. carotovora* subsp. *carotovora* (Chatterjee et al. 2002). To study the influence of GacA on the transcription of T3SS genes in Ech3937, the promoter regions of *dspE*, *hrpA*, and *hrpN* of the bacterium were cloned into the pPROBE-AT to

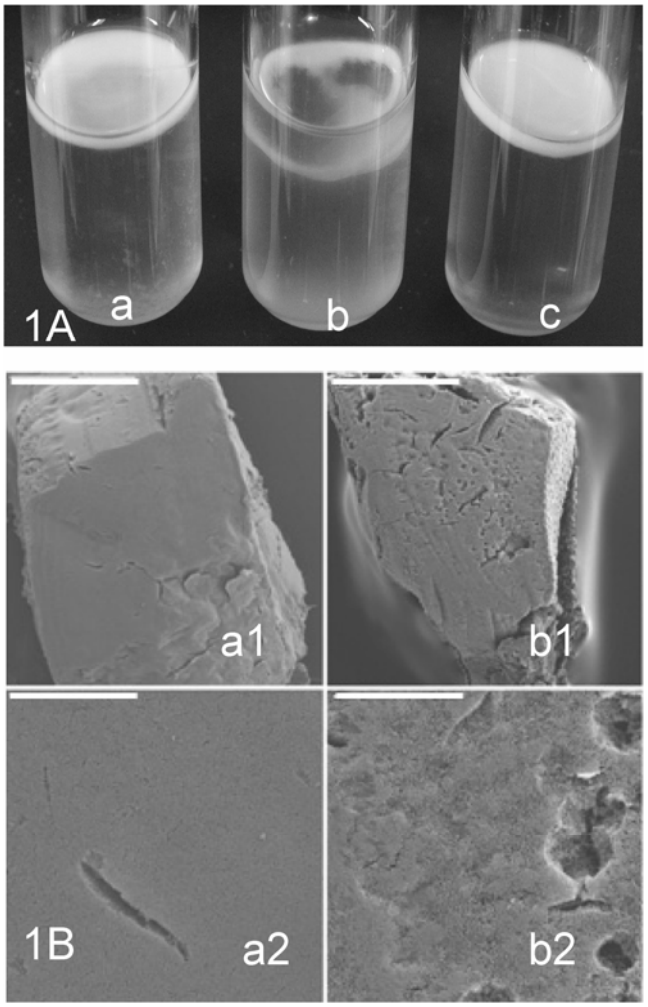

Fig. 1. A, Biofilm and pellicle formation in SOBG (Yap et al. 2005). **a,** Biofilm and pellicle formed in wild-type Ech-Rif in SOBG cultures grown for 3 days at 28°C. **b,** Delayed biofilm and pellicle formation in Ech137, the *gacA* mutant in SOBG cultures grown for 3 days at 28°C. **c,** The *gacA* gene expressed on plasmid pCLgacA restored biofilm and pellicle formation to the *gacA* mutant Ech137 in SOBG cultures grown for 3 days at 28°C. **B,** Cross sections of the pellicle observed with scanning electron microscopy at different magnifications. **a1,** Ech-Rif; **b1,** Ech137; **a2,** Ech-Rif; **b2,** Ech137. Size bar in the micrograph: a1 and b1, 1 mm; a2 and b2, 100 μm.

produce plasmids pDspE, pHrpA, and pHrpN (Table 1). The total GFP intensity of Ech-Rif (pDspE) was 15 ± 0.8 at 8 h and 17 ± 1.2 at 12 h grown in *hrp*-inducing MM (Table 2). A lower total GFP intensity of Ech137 (pDspE) of 3.4 ± 0.01 and 3.4 ± 0.05 was observed at the same period of time. Similarly, compared with Ech-Rif, a lower GFP intensity was observed in the Ech137 cells carrying pHrpA and pHrpN grown in the MM at 8 and 12 h (Table 2). The above data provide evidence that GacA upregulated *dspE*, *hrpA*, and *hrpN*.

The Gac-Rsm regulatory network controls Pel and T3SS gene expression.

Rsm is a novel type of post-transcriptional regulatory system that plays a critical role in gene expression. To investigate whether the influence of GacA on pectinase gene expression is through the Rsm regulatory pathway, the relative mRNA level of *rsmC*, *rsmB*, and *rsmA* was examined by qRT-PCR. The qRT-PCR data were analyzed using the Relative Expression Software Tool as described by Pfaffl and associates (Pfaffl et al. 2002). Compared with wild-type, a lower amount of *rsmB* mRNA was observed in Ech137. Wild-type Ech-Rif produced approximately 10-fold more *rsmB* mRNA than *gacA* mutant Ech137 at 6 h and 24-fold more at 12 h, with a *P* value less than 0.05. No significant differences in amount of *rsmC* and *rsmA* mRNA were observed between Ech-Rif and Ech137 (with a *P* value range from 0.74 to 1) (Fig. 5A). No detectable mRNA of *gacA* was observed in Ech137 by qRT-PCR (Fig. 5B). The *gacA* and *rsmB* expression of Ech137 was restored by introducing the plasmid pCLgacA into the mutant. The relative mRNA amounts of *gacA* and *rsmB* of Ech137 (pCLgacA) are approximately 180 and 150% of the Ech-Rif (Fig. 5B). The higher amounts of *gacA* and *rsmB* mRNAs in Ech137 (pCLgacA) compared with Ech-Rif may be due to the copy number effect of the plasmid.

To further investigate whether the influence of GacA on T3SS gene expression is through the GacA-RsmA-*rsmB*-*hrpL* regulatory pathway, the amount of *hrpL* mRNA of the bacteria was examined by qRT-PCR. Compared with Ech-Rif (normalized to 1), a significantly lower *hrpL* mRNA was observed in *gacA* mutant Ech137 (0.362 ± 0.065) with a *P* value of 0.001 at 12 h grown in MM (Fig. 5A). A similar amount of *hrpL* mRNA was observed between Ech-Rif and Ech137at 6 h (*P* = 1) grown in the medium. Similar promoter activity of *hrpL* was observed between Ech-Rif and Ech137 (data not shown), suggesting that *hrpL* was regulated at a post-transcriptional level.

GacA influences the expression of Pel and T3SS genes in planta.

To connect the in vitro result with the in vivo condition, the expression of *pelL* and *dspE* between Ech-Rif and Ech137 in host plant African violet (*Saintpaulia ionantha*) leaves was examined further. Compared with Ech137, a higher transcription of *pelL* and *dspE* in Ech-Rif in *S. ionantha* was observed at 24 h postinoculation, which is approximately threefold more for *pelL* and fourfold more for *dspE* (Table 3).

The *gacA* mutant reduced maceration and systemic invasion ability.

Because GacA affects multiple phenotypes contributing to pathogenesis, a local maceration assay was carried out with Ech-Rif, Ech137, and the complemented strain Ech137

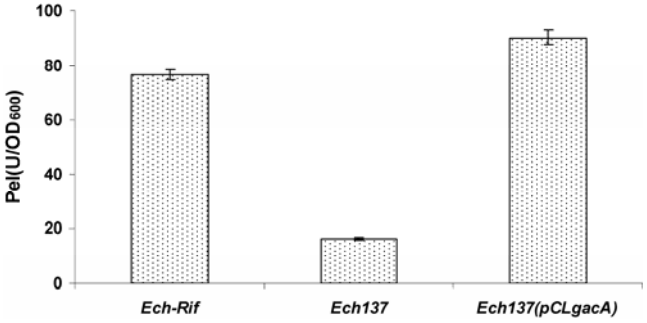

Fig. 3. Spectrophotometric quantification of pectate lyase (Pel) activity (U/optical density at 600 nm [OD_{600}]) in Ech-Rif, *gacA* mutant Ech137, and the complementary strain Ech137 (pCLgacA) grown in minimal medium supplemented with 1% polygalacturonate at 36 h was examined as described (Matsumoto et al. 2003). Values are a representative of two experiments. Three replicates were used in this experiment; the value is present as average of three replicates and the standard deviation.

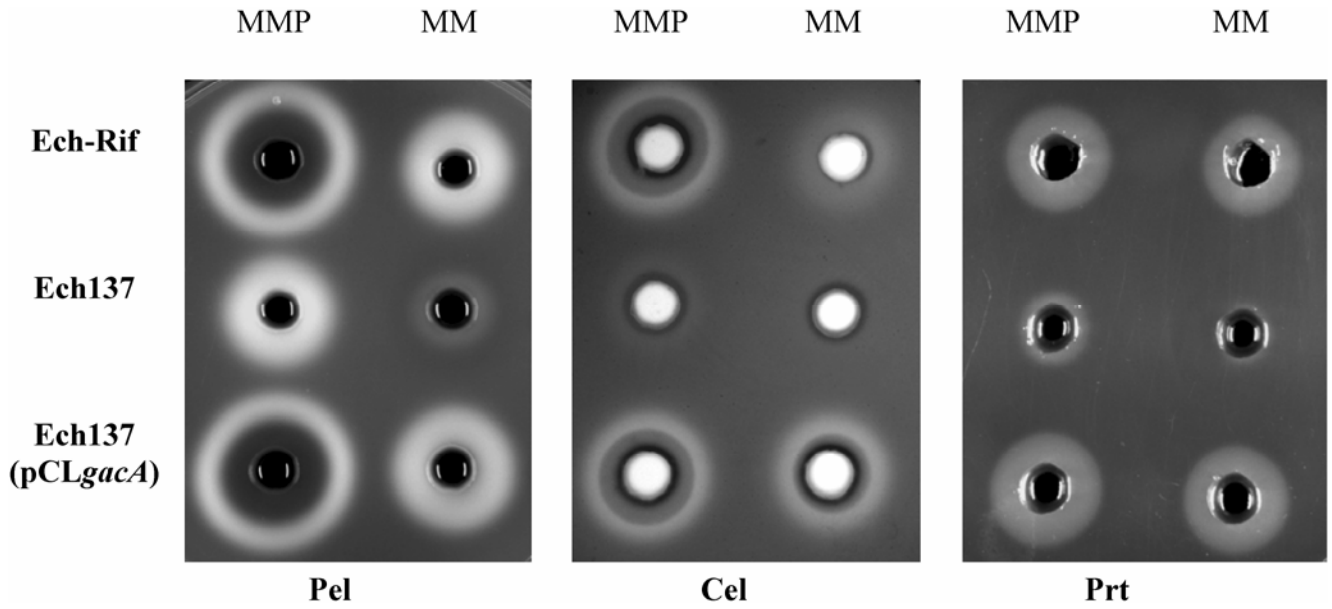

Fig. 2. Pectate lyase (Pel), protease (Prt), and cellulase (Cel) production of wild-type Ech-Rif, *gacA* mutant Ech137, and *gacA* mutant complemented strain Ech137 (pCLgacA) grown in minimal medium (MM) and MM supplemented with 1% polygalacturonate (MMP) at 36 h was examined by plate assays as described (Masumoto et al. 2003). Values are a representative of two experiments. Three replicates were used in this experiment.

(pCLgacA) in the African violet cv. Gauguin as previously described (Yang et al. 2002). Compared with Ech-Rif, Ech137 was dramatically reduced in maceration ability in planta 2 days postinoculation (Fig. 6). The maceration ability of Ech137 was restored to near the wild-type Ech-Rif level by pCLgacA (Fig. 6). Interestingly, although a lower Pel activity also was observed in plant leaves inoculated with Ech137, in comparison with the leaves inoculated with wild-type Ech-Rif (Fig. 7), there was no difference in bacterial concentration between the wild-type Ech-Rif and Ech137 in African violet leaves at day 2 postinoculation analyzed by a paired sample *t* test ($P = 0.23$) (Fig. 7). Compared with Ech-Rif, a lower bacterial concentration of Ech137 in plants was observed at day 3 ($P = 5.9 \times 10^{-11}$) and day 4 ($P = 1.7 \times 10^{-4}$).

A systemic invasion assay (Franza et al. 1999) was further applied to investigate the role of GacA of the bacterium in *S. ionantha*. Eight days after inoculation, 11 of the 12 plants inoculated with Ech-Rif developed systemic invasion symptoms (Fig. 8). In contrast, the Ech137 showed a reduced ability to develop a systemic invasion in the plant host; only one plant developed a systemic invasion with the *gacA* mutant 16 days postinoculation.

DISCUSSION

In the work of Yap and associates, no pellicle formation was observed in *hrpL*, *hrpN*, and *hrpA* mutant strains of Ech3937 (Yap et al. 2005). In this work, a delayed pellicle formation was observed in Ech137. This delayed pellicle formation phenotype suggests that *gacA* mutation delayed but did not totally eliminate the expression of T3SS genes that are essential for pellicle formation of Ech3937. Our scanning electron microscopy (SEM) observation further suggested a more loose structure of pellicle in Ech137 compared with Ech-Rif. Because cellulose is part of the *D. dadantii* pellicle (Yap et al. 2005), cellulase treatment was used for further experimentation. Pellicles of both Ech-Rif and Ech137 treated with cellulase disintegrated. This result is consistent with the report of Yap and associates (2005), who found that pellicle formation still occurs in a mutant with an insertion at the cellulose synthase subunit, suggesting that T3SS but not the cellulose synthase subunit is required for Ech3937 pellicle formation (Yap et al. 2005).

HrpL is an alternative sigma factor that activates the expression of genes encoding the T3SS and its secreted effector proteins (Fouts et al. 2002; Yap et al. 2005, 2006). From our recent microarray study of Ech3937, the transcriptome profiles of wild-type Ech3937 and the *hrpL* mutant also showed that HrpL upregulated several T3SS genes, including *dspE*, *hrpA*, and *hrpN* (*unpublished data*). There is no difference in promoter activity of *hrpL* observed between Ech-Rif and Ech137 (data not shown); therefore, the lower promoter activities of pDspE, pHrpA, and pHrpN in Ech137 found in this study may be due partially to the reduction of intact *hrpL* RNA present in

Table 2. Expression of *dspE, hrpA*, and *hrpN* of Ech-Rif and Ech137 grown in minimal medium

	Mean fluorescence intensity[a]	
Gene promoter	**8 h**	**12 h**
Ech-Rif (pDspE)	15.3 ± 0.8	17.0 ± 1.2
Ech137 (pDspE)	3.4 ± 0.01	3.4 ± 0.05
Ech-Rif (pHrpA)	25.9 ± 1.6	39.0 ± 5.0
Ech137 (pHrpA)	5.4 ± 0.2	6.6 ± 0.1
Ech-Rif (pHrpN)	21.3 ± 1.8	38.9 ± 2.4
Ech137 (pHrpN)	2.6 ± 0.05	2.7 ± 0.04

[a] Promoter activities were compared after 8 and 12 h of culture in the minimal medium. Values represent total green fluorescent protein intensity and are a representative of two experiments. Three replicates were used in this experiment. The value is present as average of three replicates and the standard deviation.

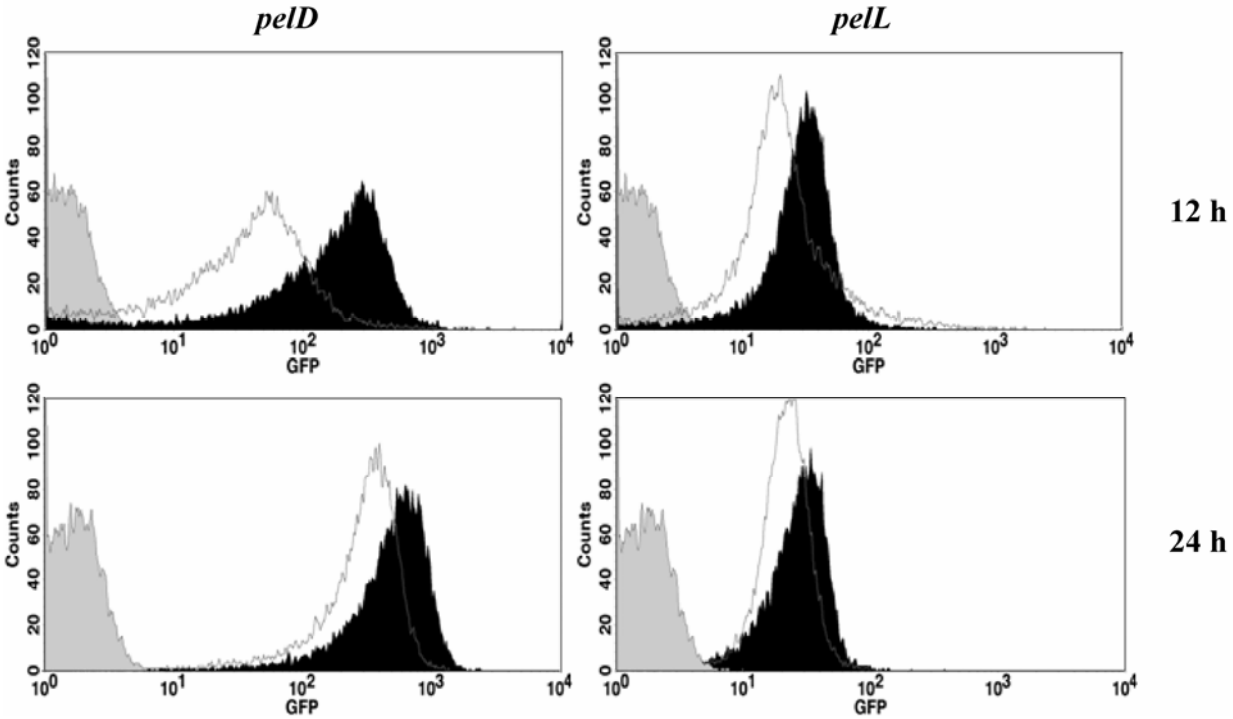

Fig. 4. Expression of *pelD* and *pelL* in Ech-Rif (black line with black filling) and *gacA* mutant Ech137 (gray line) grown in minimal medium supplemented with 1% polygalacturonate (MMP). The promoter activities were compared after 12 and 24 h of culture in the medium MMP. Green fluorescent protein (GFP) intensity was determined on gated populations of bacterial cells by flow cytometry and analyzed with the Cell Quest software (BD Biosciences, San Jose, CA, U.S.A.). The gray line with gray filling stands for the GFP expression control base level of the Ech-Rif containing pPROBE-AT vector without insert. Values are a representative of two experiments. Three replicates were used in this experiment and one replicate was used for the overlay as displayed.

the *gacA* mutant (Fig. 5A) through the post-transcriptional regulation of GacA-Rsm pathway (Chatterjee et al. 2002; Cui et al. 2001).

In *E. carotovora* subsp. *carotovora*, an RNA-binding protein, RsmA, binds to and induces the degradation of *hrpL* mRNA (Chatterjee et al. 2002; Cui et al. 2001). The degradation of *hrpL* mRNA by RsmA can be neutralized when the regulatory *rsmB* mRNA is present and bound to RsmA. The expression of *rsmB* gene is controlled by the GacA. Unlike *E. carotovora* subsp. *carotovora*, a lower amount of *hrpL* mRNA in the *gacA* mutant strain than in the wild-type Ech-Rif was observed only at 12 h grown in MM (Fig. 5A). It is unclear whether the minor effect of GacA on *hrpL* in Ech3937 is due to the lack of an effect on RsmA levels in the GacA mutant Ech137 or is due to lower expression of *hrpL* in wild-type Ech3937 than in *E. carotovora* subsp. *carotovora*.

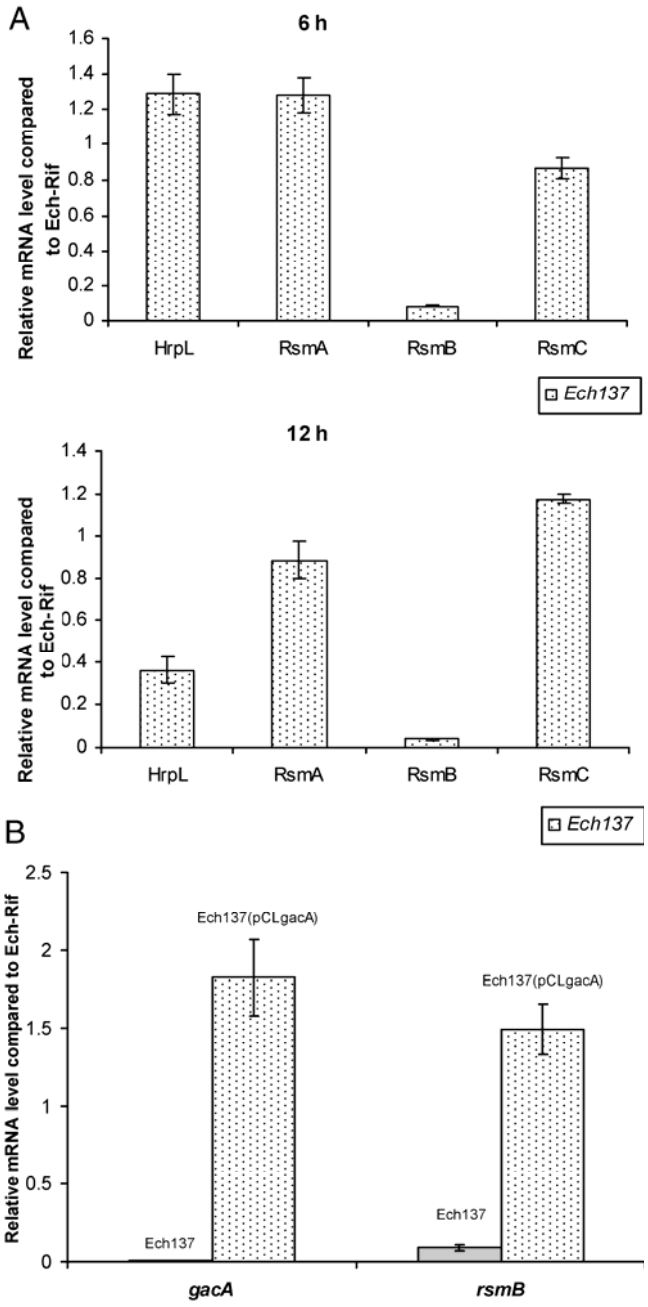

Fig. 5. A, Relative level of *rsmA*, *rsmB*, *rsmC*, and *hrpL* mRNA in *gacA* mutant Ech137 compared with wild-type Ech-Rif grown for 6 or 12 h in a minimal medium. Values are a representative of three experiments. Three replicates were used in this experiment, the value is present as average of three replicates and the standard deviation. **B,** Relative level of *gacA* and *rsmB* mRNA in *gacA* mutant Ech137 and *gacA* mutant complemented strain Ech137 (pCLgacA) compared with wild-type Ech-Rif grown for 12 h in a minimal medium. The amount of mRNA was examined by real-time polymerase chain reaction assay and analyzed by Relative Expression Software Tool as described by Pfaffl and associates (2002). The normalized value of mRNA for wild-type was 1.0. Three replicates were used in this experiment; the value is present as average of three replicates and the standard deviation.

Similar to the work of Cui and associates (2001) in *E. carotovora* subsp. *carotovora*, the production of Prt, Cel, and Pel was reduced in the *gacA* mutant of *D. dadantii.* In *E. carotovora* subsp. *carotovora*, RsmA negatively regulated the production of exoenzymes. In this study, compared with Ech-Rif, a lower *rsmB* level was observed in the *gacA* mutant Ech137. A reduced production of exoenzymes, and the lower Pel gene *pelD* and *pelL* expression in Ech137 than Ech-Rif, observed in this study (Figs. 2, 3, and 4) may be due partially to a higher free RsmA amount released from *rsmB* binding in the *gacA* deletion mutant (Fig. 5A).

In *D. dadantii*, the pectinases are considered to be the major virulence factor for host infection (Hugouvieux-Cotte-Pattat et al. 1996) and the T3SS also is required for full virulence in plant hosts (Yang et al. 2002, 2004). In addition to causing local maceration on infected leaves, the bacterium may enter vascular tissue and move through the host to cause a systemic maceration symptom (Franza et al. 1999). Our in planta virulence assay suggests that the GacA of *D. dadantii* may play the role of enhancing systemic invasion in the host plants (Fig. 8). In this study, the reduction of local maceration and systemic invasion symptoms in African violet leaves infected with Ech137 (Figs. 6 and 8) might be due to the lower expression of *pel* and T3SS genes of the bacterium in planta (Table 3).

In conclusion, *D. dadantii* usually is described as a brute-force pathogen. However, our data suggest that the bacterium might manage its nutrient uptake, multiplication, and in planta fitness through a sophisticated regulation system involving the TCSTS receptor GacA to influence virulence mechanisms such as exoenzyme production and T3SS gene expression during its invasion process. Although a lower expression of pectinase and T3SS genes of *gacA* mutant Ech137 does not affect the concentration of *D. dadantii* in *S. ionantha* in the early stage of infection, these subtle effects regulated by GacA are essential for a full establishment of the bacterium in plant hosts and the systemic movement of the bacteria. Finally, our results here demonstrate that, during the bacterial infection process, a dynamic regulation and fine tuning of several virulence factors through GacA, and possibly other corresponding regulators, are crucial for the host–pathogen interaction of *D. dadantii*. The conservation of the TCSTS GacS/GacA among *D. dadantii* and other plant pathogens on the regulation of various pathogenic determi-

Table 3. Expression of *pelL* and *dspE* of Ech-Rif and *gacA* mutant Ech137 in African violet[a]

Gene promoter	Ech-Rif (pPelL)	Ech137 (pPelL)	Ech-Rif (pDspE)	Ech137 (pDspE)
Total	51.4 ± 20.91	11.8 ± 7.4	6.0 ± 0.8	1.2 ± 0.1
GFP^+ mean	78.2 ± 17.0	40.1 ± 0.7	54.4 ± 12.2	15.8 ± 15.2
GFP^+%	57.1 ± 14.9	11.6 ± 11.3	8.0 ± 0.5	0 ± 0

[a] Promoter activities were compared after 24 h of inoculation. Green fluorescent protein (GFP) intensity was determined on gated populations of bacterial cells by flow cytometry. Values are a representative of two experiments. Three replicates were used in this experiment. The value is present as average of three replicates and the standard deviation.

nants highlights the possibility of identifying the TCSTS inhibitors for designing novel antimicrobial agents targeting this crucial signaling system (Lyon et al. 2000; Matsushita and Janda 2002).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type Ech-Rif, and its mutant strains were stored at –80°C in 15% glycerol and grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001) and MM medium (Yang et al. 2007). Antibiotics were added to the media at the following concentrations: kanamycin, 50 μg/ml; rifampicin, 100 μg/ml; ampicillin, 100 μg/ml; and spectinomycin, 50 μg/ml. The *gacA* deletion mutant was constructed by a cross-over PCR mutagenesis approach as described (Yang et al. 2002); the primers used were gacA_A, 5′ GCA CCC GAT TGC CTG TAC TTA3′; gacA_B, 5′ GCA CCA GTT CAT GGT CAT CAA C3′; gacA_C, 5′ CGG AGA CAT TGA TTA GTA GTG A3′; and gacA_D, ATT GGG AAA CGG GCC GAA GT.

GFP reporter plasmid construction.

The GFP promoter region of *dspE* and *pelD* cloned into the reporter plasmid pPROBE-AT (Leveau and Lindow 2001) was constructed previously (Peng et al. 2006). The DNA fragments of promoter regions of *hrpA*, *hrpN*, *hrpL*, and *pelL* were PCR amplified from Ech3937 chromosomal DNA and ligated into the pCR2.1-TOPO TA cloning vector system (Invitrogen, Carlsbad, CA, U.S.A.). The primer pair used for *pelL* promoter in this study is PpelL_F, 5′ATG CGG TAA TGC GGG GAT3′ and PpelL_R, 5′GGC CAG AAC TGA TGT ACT GT3′, which produces a 609-bp *pelL* promoter region sequence of Ech-Rif. The inserted DNA was further subcloned into the *Xba*I/*Sac*I sites of the promoter-probe vector pPROBE-AT (Table 1). A plasmid pCLgacA containing a full-length *gacA* in plasmid pCL1920 also was constructed using the primer set gacAco_F, 5′GCC AAT GTT TCG GGT GTA G3′ and gacAco_R, 5′CAT CGA TCT GCC GGA TAC TTT3′.

The GFP reporter in combination with the FACS-based approach has been used to evaluate gene activity in several bacteria at the single-cell level (Rietsch and Mekalanos 2006). Because the *gfp* gene in the pPROBE-AT contains its own ribosome binding site, the stability of *gfp* mRNA should not be interfered

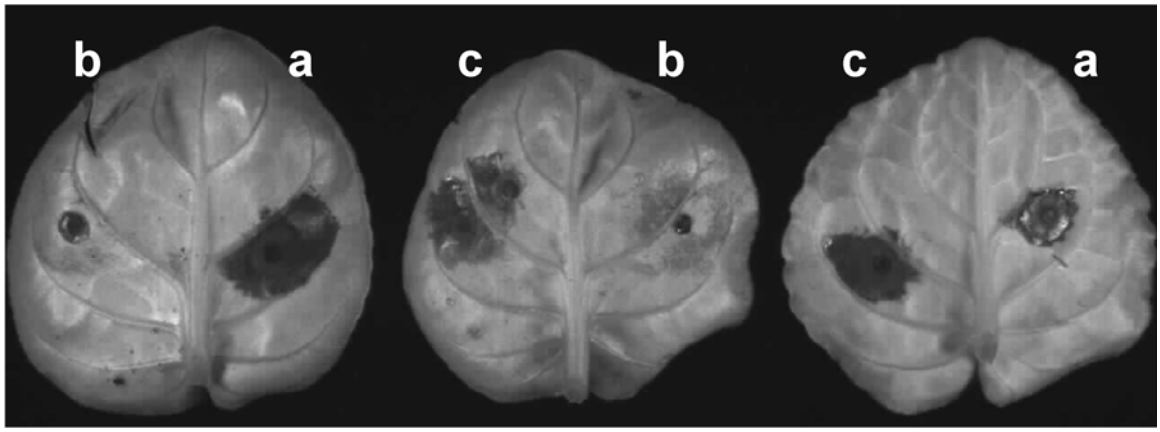

Fig. 6. Local maceration lesions caused by **a,** Ech-Rif; **b,** *gacA* mutant Ech137; and **c,** complemented strain Ech137 (pCL*gacA*). Bacterial cells were inoculated in the middle of each half side of the same leaf. Phosphate buffer (pH 7.4, 50 mM) was used to suspend the bacterial cells and a volume of a 50 μl of bacterial suspension with a bacterial concentration of 10^6 CFU/ml was used. The maceration symptom was examined 2 days postinoculation. The experiment has been repeated twice.

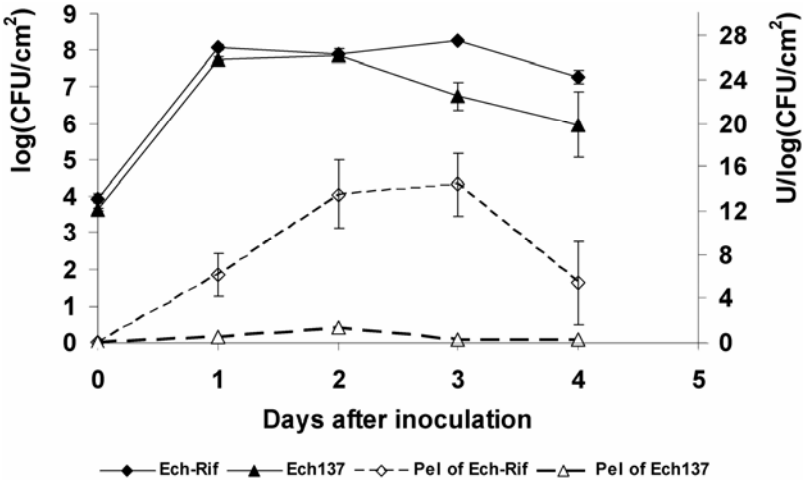

Fig. 7. Concentration of Ech-Rif and *gacA* mutant Ech137 in African violet cv. Gauguin (*Saintpaulia ionantha*). Leaves of African violet were inoculated with a 50-μl bacterial suspension at a concentration of 10^6 CFU/ml. Six leaves from six replicate plants were used at each sampling time for each bacterial strain, the value is present as average of three replicates and the standard deviation; concentration of Ech-Rif (◆) and Ech137 (▲). The spectrophotometric quantification was carried out as described to measure the pectinase (Pel) activity in the inoculated leaves from the same sample for population kinetics; Pel production of Ech-Rif (◇) and Ech137 (△).

by RsmA when a promoter-containing DNA region of Ech3937 is cloned into the reporter vector (Miller et al. 2000).

FACS investigation of promoter activity.

The bacterial cells of Ech-Rif and Ech137 carrying GFP reporter plasmid constructs were washed three times with 1× phosphate-buffered saline (PBS) buffer (8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter, pH 7.2 to 7.4) and diluted to approximately 10^6 CFU/ml before analysis. Bacterial cells were identified based on forward and side light scatter properties and electronically gated for analysis. The promoter activity was determined by FACS (Becton Dickinson, San Jose, CA, U.S.A.) and the flow cytometry results were analyzed using Cell Quest software (BD Biosciences, San Jose, CA, U.S.A.).

Real-time PCR analysis.

Wild-type Ech-Rif and the *gacA* mutant Ech137 were grown in MM with glucose as carbon source (Yang et al. 2007). Total RNA from the bacteria was isolated by using TRI reagent method (Sigma-Aldrich, St. Louis) and treated with Turbo DNA-free DNase kits (Ambion, Austin, TX, U.S.A.). An iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.) was used to synthesize cDNA from 0.5 µg of treated total RNA. The Real Master Mix (Eppendorf, Westbury, NY, U.S.A.) was used for real-time PCR reaction to quantify the cDNA level of *hrpL*, *rsmA*, *rsmB*, *rsmC*, and *rplU* in different samples. The *rplU* was used as the endogenous control for data analysis. The primer pairs used in this study were RplUsF, 5′ GCG GCA AAA TCA AGG CTG AAG TCG 3′ and RplUsR, 5′ CGG TGG CCA GCC TGC TTA CGG TAG 3′ for *rplU*; HrpLsF, 5′ GAT GAT GCT GCT GGA TGC CGA TGT 3′ and HrpLsR, 5′ TGC ATC AAC AGC CTG GCG GAG ATA 3′ for *hrpL*; rsmAf, 5′ TTT TGA CTC GTC GAG TTG GCG AAA 3′ and rsmAr, 5′ GCG CGT TAA CAC CGA TAC GAA CCT 3′ for *rsmA*; rsmBf, 5′ AGA GGG ATC GCC AGC AAG GAT TGT 3′ and rsmBr, 5′ CGT TTG CAG CAG TCC CGC TAC C 3′ for *rsmB*; and rsmCf, 5′ ACG AAG TGC TCC CGG TTA ATG TCC 3′ and rsmCr, 5′ ACG AGA GCG TAC TGA GCG GCT TTT 3′ for *rsmC*. Reactions were run and data were collected by the Opticon 2 system (Bio-Rad). Real-time PCR data were analyzed using Relative Expression Software Tool as described by Pfaffl and associates (2002).

Pellicle formation and exoenzyme production.

For pellicle formation assay, bacterial strains were grown in SOBG broth at 28°C as described (Yap et al. 2005). Due to the slow formation of pellicle in Ech137, 10-day-old pellicles from Ech-Rif and Ech137 were used for SEM observation. The samples of pellicle were fixed in 2% glutaraldehyde in PBS buffer (pH 7.0) for 2 h and post-fixed in 1% osmium tetroxide in the same buffer for 1 h. After dehydration in the graded series of ethanol, specimens were infiltrated with polyethylene glycol (PEG). Cross sections of the pellicles were cut using an ultramicrotome. Then, PEG was extracted from the blocks by soaking in several changes of warm ethanol. After critical-point drying, the specimen was mounted on a stub coated with Duco cement, sputter coated with gold, and examined with a Hitachi S-570 Scanning Electron Microscope.

Plate assays for activity of Pel, Cel, and Prt and the spectrophotomeric assay of Pel activity for Ech-Rif, Ech137, and the complemented strain Ech137 (pCLgacA) were carried out as described by Matsumoto and associates (2003). Three biological replicates were performed for each treatment.

Virulence assay, growth kinetics, and in planta Pel production.

The local leaf maceration assay was carried out as described (Yang et al. 2002, 2004). Briefly, wild-type bacterial cells and *gacA* mutant Ech137 cells were syringe infiltrated in the middle of each symmetric side of the same leaf with approximately 50 µl of a bacterial suspension at 10^6 CFU/ml. Phosphate buffer (50 mM, pH 7.4) was used to suspend the bacterial cells. Three replicate plants with a total of at least 12 leaves were inoculated. In the systemic invasion assay, the pathogenicity of the bacterium was evaluated as described by Franza and associates (1999), with minor modification. A volume of a 50 µl of the bacterial suspension with an optical density at 600 nm of 0.01 was inoculated into the front edge of the African violet leaf. For each bacterial strain, 12 plants were inoculated. Inoculated plants were kept in growth chambers at 28°C, 95% relative humidity, and a photoperiod of 16 h. Development of symptoms induced by bacterial strains in African violet plants was considered as systemic when at least one leaf and its petiole were macerated. Progression of the symptoms was scored daily for 12 days.

Growth kinetics in planta was carried out in African violet cv. Gauguin as described (Yang et al. 2002). Briefly, leaves

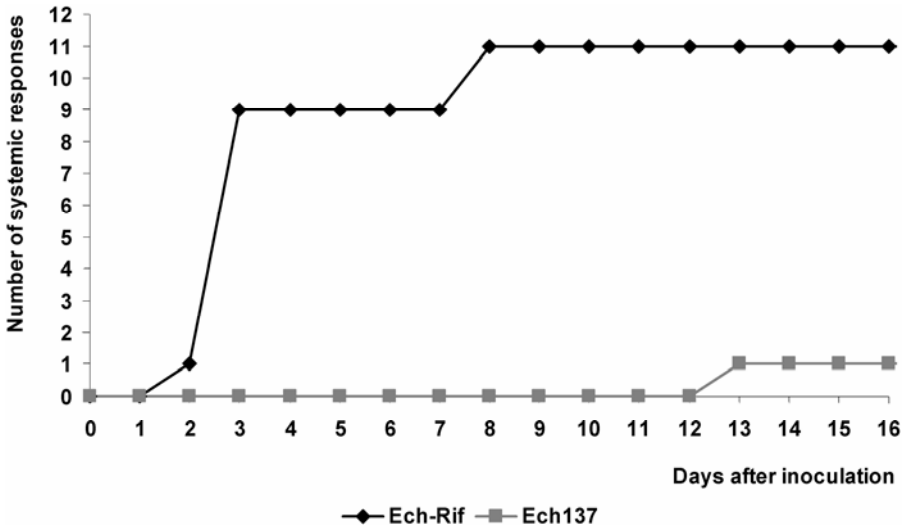

Fig. 8. Development of symptoms induced by Ech-Rif and Ech137 strains in African violet cv. Gauguin (*Saintpaulia ionantha*) plants. A systemic invasion assay was used as described by Franza and associates (1999) with minor modification. For each strain, 12 plants (one leaf per plant) were inoculated. Response was considered as systemic when at least one leaf and its petiole were macerated. Values are a representative of two experiments.

were syringe infiltrated with approximately 50 µl of bacterial suspension at 10^6 CFU/ml with a 1-ml syringe. Leaf discs (4 mm in diameter) around the maceration area were harvested at different intervals following infiltration and ground in 50 mM phosphate buffer (pH 7.4). The bacterial concentration, (CFU/cm^2) was determined by plating serial dilutions of leaf extracts on LB agar plates. A spectrophotomeric assay was used to monitor the Pel production of Ech-Rif and Ech137 during the in planta growth. A 10-µl supernatant of the plant juice from African violet leaves inoculated with the bacteria was added into 990 µl of Pel reaction buffer and the Pel production was quantified using the spectrophotomeric assay (Matsumoto et al. 2003). Pel production was the ratio of the optical density at 230 nm unit to the log unit of the bacterial concentration (U/log [CFU/cm^2]). Three replicate plants with a total of six leaves per plant were used in each sampling time for the in planta Pel production and bacterial growth kinetics assays.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

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asap.ahabs.wisc.edu/asap/ASAP1.htm