Effects of the Two-Component System Comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the Production of Global Regulatory *rsmB* RNA, Extracellular Enzymes, and Harpin_{Ecc}

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Posttranscriptional regulation mediated by the regulator of secondary metabolites (RSM) RsmA-*rsmB* pair is the most important factor in the expression of genes for extracellular enzymes and Harpin_{Ecc} in *Erwinia carotovora* subsp. *carotovora*. RsmA is a small RNA-binding protein, which acts by lowering the half-life of a mRNA species. *rsmB* specifies an untranslated regulatory RNA and neutralizes the RsmA effect. It has been speculated that GacA-GacS, members of a two-component system, may affect gene expression via RsmA. Because *expA*, a *gacA* homolog, and *expS* (or *rpfA*), a *gacS* homolog, have been identified in *E. carotovora* subsp. *carotovora*, we examined the effects of these *gacA* and *gacS* homologs on the expression of *rsmA*, *rsmB*, and an assortment of exoprotein genes. The *gacA* gene of *E. carotovora* subsp. *carotovora* strain 71 stimulated transcription of genes for several extracellular enzymes (*pel-1*, a pectate lyase gene; *peh-1*, a polygalacturonase gene; and *celV*, a cellulase gene), *hrpN_{Ecc}* (an *E. carotovora* subsp. *carotovora* gene specifying the elicitor of hypersensitive reaction), and *rsmB* in GacA⁺ and GacS⁺ *E. carotovora* subsp. *carotovora* strains. Similarly, the *E. carotovora* subsp. *carotovora* *gacA* gene stimulated *csrB* (*rsmB*) transcription in *Escherichia coli*. A GacS⁻ mutant of *E. carotovora* subsp. *carotovora* strain AH2 and a GacA⁻ mutant of *E. carotovora* subsp. *carotovora* strain Ecc71 compared with their parent strains produced very low levels of *rsmB*, *pel-1*, *peh-1*, *celV*, and *hrpN_{Ecc}* transcripts but produced similar levels of *rsmA* RNA and RsmA protein as well as transcripts of hyperproduction of extracellular enzymes (Hex) *hexA*, *kdgR* (repressor of genes for uronate and pectate catabolism), *rsmC*, and *rpoS* (gene for Sigma-S, an alternate Sigma factor). The levels of *rsmB*, *pel-1*, *peh-1*, *celV*, and *hrpN_{Ecc}* transcripts as well as production of pectate lyase, polygalacturonase, cellulase, protease, and Harpin_{Ecc} proteins were stimulated in GacS⁻ and GacA⁻ mutants by GacS⁺ or GacA⁺ plasmids, respectively. The GacA effect on exoenzyme genes and *hrpN_{Ecc}* was abrogated in *E. carotovora* subsp. *carotovora* mutants deficient in RsmA and RsmC or RsmA, RsmC, and *rsmB* RNA. The expression of *lacZ*

transcriptional fusions of *rsmB* of *Erwinia amylovora* and *Erwinia herbicola* pv. *gypsophylae* was markedly reduced in a GacA⁻ and a GacS⁻ mutant of *Pseudomonas syringae* pv. *syringae*. Southern blot hybridization revealed the presence of *gacA* and *gacS* homologs in all tested strains of soft-rotting *Erwinia* spp. and several nonsoft-rotting *Erwinia* species such as *E. amylovora*, *E. rhapontici*, *E. herbicola*, *E. stewartii*, and *E. herbicola* pv. *gypsophylae*. These findings establish that the GacA-GacS system controls transcription of *rsmB* of *E. carotovora* subsp. *carotovora*, *E. amylovora*, and *E. herbicola* pv. *gypsophylae* and support the hypothesis that the effects of this two-component system on extracellular protein production in *E. carotovora* subsp. *carotovora* is mediated, at least in part, via the levels of *rsmB* transcripts.

Erwinia carotovora subsp. *carotovora* produces an array of extracellular proteins (exoproteins) that are secreted via three distinct secretion systems (Salmond 1994). Examples include protease (Prt) secreted by the type I system; pectate lyase (Pel), polygalacturonase (Peh), and cellulase (Cel) secreted by the type II system; and Harpin_{Ecc} secreted by the type III system. Although secreted by different pathways, these exoproteins are coregulated by plant signals, the quorum sensing signal, N-acyl homoserine lactone, various transcriptional factors as well as posttranscriptional regulators (Andersson et al. 1999; Barras et al. 1994; Chatterjee et al. 1995; Chatterjee et al. 2000; Cui et al. 1995; Cui et al. 1999; Harris et al. 1998; Jones et al., 1993; Liu et al. 1998; Liu et al. 1999; Mukherjee et al. 1997; Mukherjee et al. 2000; Pirhonen et al. 1993; Shih et al. 1999; Thomson et al. 1999). Of these, posttranscriptional regulation mediated by the regulator of secondary metabolites (RSM) RsmA-*rsmB* RNA is the most important parameter (Chatterjee et al. 1995; Cui et al. 1995; Liu et al. 1998). RsmA is a small RNA binding protein that causes decay of RNA species and behaves as a global negative regulator. *rsmB* RNA binds RsmA and neutralizes its negative effect. It is believed that the relative levels of RsmA, *rsmB* RNA, and the *rsmB* RNA-RsmA ribonucleoprotein complex largely determine whether the genes for exoproteins would be expressed or remain suppressed.

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Previous studies have revealed that several transcriptional factors control exoprotein production in *E. carotovora* subsp. *carotovora* (Cui et al. 1999; Liu et al. 1998; Liu et al. 1999; Mukherjee et al. 2000; Thomson et al. 1999). HexA (Harris et al. 1998; Mukherjee et al. 2000), a LysR like protein (Schell 1993), negatively regulates extracellular enzymes and Harpin_{Ecc} production mainly by affecting the levels of RsmA and *rsmB* RNA (Mukherjee et al. 2000). Similarly, RsmC affects production of these proteins by modulating the levels of RsmA and *rsmB* RNA (Cui et al. 1999). KdgR, an IclI-like protein, negatively regulates Pel and Peh production by binding to their respective operator regions and affects the production of these and other exoproteins (i.e., Cel, Prt, and harpin) by inhibiting the transcription of *rsmB* by a novel roadblock mechanism (Liu et al. 1999). The work of A. Handa and T. Palva and their associates documented that members of the two-component system ExpS, a homolog of GacS, and ExpA, a homolog of GacA, control extracellular enzyme production and pathogenicity in *E. carotovora* subsp. *carotovora* strains (Eriksson et al. 1998; Frederick et al. 1997). How this regulation is brought about, however, was not clarified.

The effects of GacS–GacA or similar two-component regulatory systems have been examined in *Pseudomonas syringae* pv. *syringae* (Barta et al. 1992; Hrabak and Willis 1992; Hrabak and Willis 1993; Rich et al. 1994), *Pseudomonas viridiflava* (Liao et al. 1994; Liao et al. 1996), *Pseudomonas fluorescens* (Corbell and Loper 1995; Lam et al. 1994; Laville et al. 1992; Whistler et al. 1998), and various other bacteria (Dzjeiamn and Mekalanos 1996). It is now apparent that these regulators or their homologs occur in many prokaryotes as well as eukaryotes and control diverse prokaryotic phenotypes, including pathogenicity (Altier et al. 2000; Grewal et al. 1995; Johnston et al. 1996; Rahme et al. 1995; Wong et al. 1998; Zhang and Normark 1996). Similar to ExpA–ExpS, however, the mechanism by which gene activation occurs was, until now, not known. A recent work in *P. fluorescens* raised the possibility that the GacA effects entails neutralization of the RsmA action (Blumer et al. 1999; Blumer et al. 2000). Because *rsmB* RNA binds RsmA and neutralizes its negative effect, we considered the possibility that ExpA and ExpS (hereafter GacA and GacS, respectively) actually regulates *rsmB* transcription, and the effect on exoprotein production is a result of this regulation. We present here data to support this prediction and show that *hrpN*_{Ecc} expression in *E. carotovora* subsp. *carotovora* also is controlled by the GacA–GacS system, which contrasts with the absence of an effect of these regulators on *hrp* expression in *P. syringae* pv. *syringae* (Willis et al. 1990). Our data establish that in *Erwinia* spp., the GacA–GacS effect on extracellular protein production is mediated via the posttranscriptional regulatory system.

RESULTS

Identification of *gacA* and *gacS* loci in *E. carotovora* subsp. *carotovora* strain 71 (Ecc71).

Previous studies have documented that the homologs of the two-component regulators *gacA* (*expA*) and *gacS* (*expS* or *rpfA*) positively control several extracellular enzymes and virulence in *E. carotovora* subsp. *carotovora* (Eriksson et al. 1998; Frederick et al. 1997). To examine the effects of *gacA*

and *gacS* on other global regulatory systems that have since been discovered, we cloned the *gacA* and *gacS* wild-type alleles of Ecc71. With the use of primers derived from the published nucleotide sequence of *E. carotovora* subsp. *carotovora* strain SCC3193, *expA* and *expS*, a 1.0-kb fragment containing the entire *gacA* gene and a 1.2-kb fragment containing 5′ segment of *gacS* were amplified by polymerase chain reaction (PCR) from chromosomal DNA of Ecc71 and cloned. Subsequently, a *gacA*⁺ (pAKC1051) and a *gacS*⁺ plasmid (pAKC1052) were obtained by screening the genomic library of Ecc71 with the PCR products as probes. The presence of appropriate genes in these plasmids was confirmed by Southern blot assays.

Plasmid pAKC1053, which contains the PCR product of *gacA* of Ecc71, was sequenced. The homology search revealed that the predicted sequence of GacA of Ecc71 is 93% identical to ExpA of SCC3193 (Eriksson et al. 1998) and 53% identical to GacA of *P. syringae* pv. *syringae* (Rich et al. 1994). It also has 78% amino acid identity to UvrY from *Escherichia coli* (Sharma et al. 1996) and 79% identity to SirA from *Salmonella typhimurium* (Johnston et al. 1996). The nucleotide sequence of the PCR product of *gacS* from Ecc71 corresponding to approximately 20 to 237 of *E. carotovora* subsp. *carotovora* strain AH2 *rpfA* (*gacS*) has 93% identity with *rpfA* of AH2 and 91% identity with *expS* (*gacS*) of SCC3193. Therefore, *gacA* and *gacS* of Ecc71 share high homology with *expA* (*gacA*) and *expS* or *rpfA* (*gacS*) of SCC3193 or AH2.

GacA and GacS positively control extracellular enzyme levels.

To determine the effects of GacA on extracellular enzyme production, the GacA⁺ plasmid pAKC1055 and the cloning vector pRK415 were transferred into Ecc71. Bacterial constructs were grown in minimal salt medium plus sucrose (0.5%, wt/vol) and tetracycline, and the culture supernatants were assayed for Pel, Peh, Cel, and Prt. The cells from these cultures were used for the isolation of total RNA for transcript assays. The results for enzymatic assays (Table 1 and Fig. 1A, columns 1 and 2) showed that the levels of Pel, Peh, Cel, and Prt were much higher in Ecc71 carrying pAKC1055 than in Ecc71 carrying the vector. Similarly, the levels of transcripts of *pel-1*, *peh-1*, and *celV* were considerably higher in Ecc71 carrying pAKC1055 than Ecc71 carrying pRK415 (Fig. 1D, columns 1 and 2). Thus, GacA positively controls extracellular enzyme production in Ecc71 by sustaining high levels of the transcripts of the cognate genes, as previously reported by Eriksson et al. (1998) for strain SCC3193.

To confirm that *gacA*⁺ DNA was responsible for this positive effect, we inactivated *gacA*⁺ DNA in pAKC1055 by inserting a Gm^r cassette in a unique *EcoRV* site within *gacA* to produce pAKC1057. pAKC1057 was then used to replace the chromosomal *gacA*⁺ allele in Ecc71. The resulting GacA[−] mutant (AC5056) has the following characteristics: i) it produced barely detectable Pel, Peh, Cel, and Prt activities (Fig. 1E, column 2, and Table 1); ii) the transcript levels of *pel-1*, *peh-1*, and *celV* in AC5056 were very low compared with the wild-type parent Ecc71 (Fig. 1F, columns 1 and 2); and iii) the GacA⁺ plasmid pAKC1055 restored the production of Pel, Peh, Cel, and Prt (Fig. 1E, column 4, and Table 1) and *pel-1*, *peh-1*, and *celV* transcripts in AC5056 (Fig. 1F, column 4).

An RpfA⁻ (GacS⁻) mutant AH2552 of AH2, pleiotropically defective in extracellular enzyme production and pathogenicity, was described by Frederick et al. (1997). We used this mutant to determine the GacS effects on extracellular enzyme production. The results in Table 1 and Figure 2A show that the levels of Pel, Peh, Cel, and Prt were much lower in AH2552 than in its parent, AH2. While the levels of transcripts of exoenzyme genes were lower in AH2552 than in AH2, the levels of *peh-1* and *celV* transcripts were reduced more severely than the levels of *pel-1* mRNA (Fig. 2C). We then transferred the GacS⁺ plasmid pAKC1052 or the cloning vector pLARF5 into AH2552 and AH2. AH2552 carrying the vector produced barely detectable levels of Pel, Peh, Cel, and Prt activities, whereas the production of these enzymes was restored by pAKC1052 in AH2552. The wild-type parent AH2 carrying pAKC1052 also produced higher levels of extracellular enzymes compared to AH2 carrying the vector, most likely a result of the dosage of the *gacS*⁺ DNA (Table 1 and Fig. 2B). The total RNA samples isolated from AH2552 and AH2 carrying pAKC1052 or pLARF5 were used to determine the levels of *pel-1*, *peh-1*, and *celV* RNAs by Northern blot analysis. The transcripts of these genes were present at higher levels in AH2 or AH2552 carrying pAKC1052 than in AH2 or AH2552 carrying pLARF5 (Fig. 2D).

Table 1. Levels of pectate lyase (Pel) produced by *Erwinia carotovora* subsp. *carotovora* (Ecc) constructs

Bacterial construct	Relevant phenotype ^a	Pel Sp act (U/ml/A ₆₀₀ unit)
Ecc71 ^b	GacA ⁺ , GacS ⁺	0.20
AC5056 ^b	GacA ⁻ , GacS ⁺	0.01
Ecc71-pRK415 ^c	GacA ⁺ , GacS ⁺ (vector)	0.07
Ecc71-pAKC1055 ^c	GacA ⁺ , GacS ⁺ (GacA ⁺)	1.01
AC5056-pRK415 ^c	GacA ⁻ , GacS ⁺ (vector)	0.01
AC5056-pAKC1055 ^c	GacA ⁻ , GacS ⁺ (GacA ⁺)	0.92
AH2 ^d	GacA ⁺ , GacS ⁺	0.45
AH2552 ^d	GacA ⁺ , GacS ⁻	0.03
AH2-pLARF5 ^e	GacA ⁺ , GacS ⁺ (vector)	0.25
AH2-pAKC1052 ^e	GacA ⁺ , GacS ⁺ (GacS ⁺)	0.45
AH2552-pLARF5 ^e	GacA ⁺ , GacS ⁻ (vector)	0.03
AH2552-pAKC1052 ^e	GacA ⁺ , GacS ⁻ (GacS ⁺)	0.44
AC5071-pRK415 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁺ (vector)	1.36
AC5071-pAKC1055 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁺ (GacA ⁺)	4.21
AC5054-pRK415 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁻ (vector)	4.70
AC5054-pAKC1055 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁻ (GacA ⁺)	4.74
AC5055-pRK415 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁻ (vector)	0.64
AC5055-pAKC1055 ^c	RsmA ⁺ RsmB ⁺ RsmC ⁻ (GacA ⁺)	0.64

^a Relevant phenotypes conferred by the plasmids are in parentheses.

^b Bacteria were grown at 28°C in minimal salts medium plus sucrose to a Klett value of approximately 200, and culture supernatants were used for enzyme assays.

^c Bacteria were grown at 28°C in minimal salts medium plus sucrose and tetracycline to a Klett value of approximately 200, and culture supernatants were used for enzyme assays.

^d Bacteria were grown at 28°C in minimal salts medium plus sucrose and celery extract to a Klett value of approximately 260, and culture supernatants were used for enzyme assays.

^e Bacteria were grown at 28°C in minimal salts medium plus sucrose, celery extract, and tetracycline to a Klett value of approximately 260, and culture supernatants were used for enzyme assays.

GacA and GacS control *hrpN*_{Ecc} expression.

Our previous studies have shown that *hrpN*_{Ecc} expression and Harpin_{Ecc} levels in Ecc71 are coregulated with extracellular enzymes. Thus, in light of the effects of GacA and GacS on extracellular enzyme production, it was of interest to examine the effects of GacA and GacS on *hrpN*_{Ecc} expression. The results (Fig. 1B, lanes 1 and 2; and D, columns 1 and 2) show that *hrpN*_{Ecc} mRNA and Harpin_{Ecc} levels were higher in Ecc71 carrying multiple copies of *gacA* than in Ecc71 carrying the cloning vector.

The total RNA samples and protein extracts from the GacS⁻ mutant AH2552 and its parent AH2 as well as the GacA⁻ mutant AC5056 and its parent Ecc71 were used to determine the levels of *hrpN*_{Ecc} mRNA and Harpin_{Ecc}. The results (Figs. 1F, columns 1 and 2; G, lanes 1 and 2; and 2C and E, lanes 1 and 2) show that *hrpN*_{Ecc} mRNA and Harpin_{Ecc} levels were barely detectable in the GacS⁻ or the GacA⁻ mutant. Northern blot analysis of the RNAs and Western blot assays with the cell extracts from the AH2552 cultures and its parent AH2 carrying the GacS⁺ plasmid pAKC1052 or the cloning vector and AC5056 and its parent Ecc71 carrying the GacA⁺ plasmid pAKC1055 or the cloning vector revealed that the levels of *hrpN*_{Ecc} transcript and Harpin_{Ecc} were restored in AH2552 carrying multiple copies of *gacS* (Fig. 2D, columns 3 and 4; and E, lanes 5 and 6) or AC5056 carrying multiple copies of *gacA* (Fig. 1F, columns 3 and 4; and G, lanes 3 and 4), whereas the levels of *hrpN*_{Ecc} mRNA and Harpin_{Ecc} were barely detectable in AH2552 or AC5056 carrying the cloning vector. The wild-type parents AH2 carrying multiple copies of *gacS* or Ecc71 carrying multiple copies of *gacA* also produced higher levels of *hrpN*_{Ecc} mRNA and Harpin_{Ecc} than these strains carrying the cloning vectors, respectively (Figs. 1B, lanes 1 and 2; D, columns 1 and 2; 2D, columns 1 and 2; and E, lanes 3 and 4).

GacA and GacS control extracellular enzyme production and expression of *hrpN*_{Ecc} via *rsmB*.

We have shown that extracellular enzyme production, *hrpN*_{Ecc} expression, and Harpin_{Ecc} levels are tightly controlled by RsmA-*rsmB* regulatory system in *E. carotovora* subsp. *carotovora* (Cui et al. 1996; Liu et al. 1998; Mukherjee et al. 1997). Because GacA and GacS positively control exoenzyme production and expression of *hrpN*_{Ecc} and Harpin_{Ecc}, we considered the possibility that GacA and GacS affected *rsmA* and *rsmB* expression, and this modulated the levels of exoproteins. The results of Northern blot analysis allow the following conclusions: i) The GacA⁻ mutant, AC5056, produced lower levels of *rsmB* transcript compared with its parent, Ecc71 (Fig. 1F, columns 3 and 4); ii) Ecc71 carrying multiple copies of *gacA* produced higher level of *rsmB* transcripts than when it carried the vector (Fig. 1D, columns 1 and 2); iii) the levels of *rsmB* transcripts were lower in the GacS⁻ mutant AH2552 than in its parent, AH2 (Fig. 2C); iv) the GacS⁺ plasmid pAKC1052 stimulated *rsmB* RNA production in the GacS⁻ mutant AH2552 (Fig. 2D, columns 3 and 4); and v) the wild-type strain AH2 carrying the *gacS*⁺ plasmid pAKC1052 compared with the vector control produced higher levels of *rsmB* RNA, most likely as a result of the dosage of the *gacS*⁺ DNA (Fig. 2D, columns 1 and 2).

rsmB genes have been cloned from several *Erwinia* species, including *E. amylovora* (designated *rsmB_{Ea}*) and *E. herbicola* pv. *gypsophylae* (designated *rsmB_{Ehg}*) (Dumenyo et al. 1999). To strengthen the findings of the positive regulatory effects of GacA and GacS on *rsmB* expression, we used *lacZ* transcrip-

tional fusions of *rsmB_{Ecc}*, *rsmB_{Ea}*, and *rsmB_{Ehg}* in a promoter probe vector, pMP220. Plasmids carrying *rsmB-lacZ* fusions or the cloning vector were transferred into *E. carotovora* subsp. *carotovora* strain AC5006 and its GacA⁻ mutant (AC5057) as well as *P. syringae* pv. *syringae* strain B728a and

Fig. 1. Effect of *gacA* on production of exoenzymes and harpin_{Ecc} and on the levels of transcripts of exoenzyme genes, *hrpN_{Ecc}*, *rsmB*, and *csrB*. *Erwinia* spp. constructs were grown in minimal salts medium plus sucrose with or without tetracycline at 28°C to a Klett value of approximately 150 for total RNA isolation and to a Klett value of 200 for enzymatic assays and Western blot analysis. *Escherichia coli* constructs were grown in Luria-Bertani medium plus tetracycline at 28°C to a Klett value of 150 for total RNA extraction. **A**, Agarose plate assays for polygalacturonase (Peh), protease (Prt), and cellulase (Cel) activities of Ecc71 carrying the cloning vector pRK415 (lane 1) or the GacA⁺ plasmid pAKC1055 (lane 2); RsmA⁻ mutant AC5071 carrying pRK415 (column 3) or pAKC1055 (column 4); RsmA⁻RsmC⁻ mutant, AC5054 carrying pRK415 (lane 5) or pAKC1055 (lane 6); and the RsmA⁻RsmC⁻RsmB⁻ mutant AC5055 carrying pRK415 (lane 7) or pAKC1055 (lane 8). Ten microliters of supernatant was used for each assay. **B**, Western blot analysis of harpin_{Ecc}. Lane 1, Ecc71-pRK415; lane 2, Ecc71-pAKC1055; lane 3, AC5071-pRK415; lane 4, AC5071-pAKC1055; lane 5, AC5054-pRK415; lane 6, AC5054-pAKC1055; lane 7, AC5055-pRK415; and lane 8, AC5055-pAKC1055. **C**, *csrB* transcripts levels in *E. coli* strain MC4100 carrying pRK415 (lane 1) and pAKC1055 (lane 2). **D**, Levels of transcripts of *pel-1*, *peh-1*, *celV*, *hrpN_{Ecc}*, and *rsmB*. Lane 1, Ecc71-pRK415; lane 2, Ecc71-pAKC1055; lane 3, AC5071-pRK415; lane 4, AC5071-pAKC1055; lane 5, AC5054-pRK415; lane 6, AC5054-pAKC1055; lane 7, AC5055-pRK415; and lane 8, AC5055-pAKC1055. **E**, Agarose plate assays for Peh, Prt, and Cel activities of Ecc71 (lane 1) and its GacA⁻ mutant AC5056 (lane 2), and AC5056 carrying the cloning vector pRK415 (lane 3) or the GacA⁺ plasmid pAKC1055 (lane 4). **F**, Levels of transcripts of *pel-1*, *peh-1*, *celV*, *hrpN_{Ecc}*, and *rsmB* in Ecc71 (lane 1), AC5056 (lane 2) and AC5056 carrying cloning vector pRK415 (lane 3) or the GacA⁺ plasmid pAKC1055 (lane 4). **G**, Western blot analysis of harpin_{Ecc} of Ecc71 (lane 1), AC5056 (lane 2), and AC5056 carrying pRK415 (lane 3) or pAKC1055 (lane 4). Each lane contained 10 µg of total bacterial protein for Western blot analysis; 10 µg of total RNA for Northern blot analysis of *pel-1*, *peh-1*, *celV*, and *hrpN_{Ecc}*; and 5 µg of total RNA for *rsmB* and *csrB*.

its GacA⁻ (BGACX) and GacS⁻ (NPS3136) mutants. The data in Figure 3A indicate that AC5057 carrying each of those fusions produced lower levels of β -galactosidase activity than its parent AC5006 carrying *rsmB-lacZ* fusions. The results in Figure 3B revealed that the expression of *rsmB_{Ea}-lacZ* and *rsmB_{Ehg}-lacZ* fusions in the GacA⁻ or GacS⁻ mutant of *P. syringae* pv. *syringae* was lower compared with the expression in the parent strain B728a carrying these fusions. Although the levels of β -galactosidase were variable depending on the source of the *rsmB* gene, these results demonstrate that GacA and GacS stimulate the expression of *rsmB* genes in the *Erwinia* species.

In *E. coli*, *csrB* specifies a regulatory RNA, which is very similar functionally to *rsmB* RNA (Liu et al. 1997). To examine the effects of multiple copies of *gacA*, we transferred pAKC1055 and the cloning vector pRK415 into MC4100 and assayed for *csrB* transcripts. The data in Figure 1C demonstrate that the *Ecc gacA* gene stimulates the production of *csrB* transcripts in *E. coli*.

We next examined the GacA and GacS effects on *rsmA* expression. Total RNAs and cell extracts from the GacA⁻ mutant

AC5056 and its parent *Ecc71* and the GacS⁻ mutant AH2552 and its parent strain AH2 were assayed. Northern blot analysis for *rsmA* mRNA and Western blot analysis for RsmA show that GacA or GacS did not affect the levels of *rsmA* RNA or RsmA protein (Fig. 4).

As stated above, extracellular protein production in *E. carotovora* subsp. *carotovora* is controlled by several other regulators such as HexA (Harris et al. 1998; Mukherjee et al. 2000), KdgR (Liu et al. 1999; Thomson et al. 1999), RsmC (Cui et al. 1999), and RpoS (Andersson et al. 1999; Mukherjee et al. 1998). To eliminate the possibility that GacS-GacA modulated the levels of these regulators, we conducted Northern analysis of total RNA samples extracted from *Ecc71* and its GacA⁻ mutant AC5056 and AH2 and its GacS⁻ mutant AH2552. The transcript levels of each of these genes were comparable in these strains (data not shown), indicating that GacS-GacA did not affect the steady-state levels of mRNAs for these regulators.

Those findings collectively support the idea that the GacS-GacA effect is channeled mostly by the regulation of *rsmB* transcription. In previous studies, we have shown that *rsmB*

Fig. 2. Effect of *gacS* on production of **A and B**, exoenzymes **E**, harpin_{Ecc} and **C and D**, on the levels of transcripts of exoenzyme genes, *hrpN_{Ecc}*, and *rsmB*. Data in **A and B** were obtained from bacteria grown at 28°C in minimal salts medium plus sucrose and celery extract to a Klett value of approximately 260; **C–E**, from bacteria grown in minimal salts medium plus sucrose to a Klett value of approximately 150. Tetracycline was added to the media for the constructs containing plasmid. Twenty microliters of each culture supernatant was used for enzymatic assays, 10 μ g of each total RNA was used for Northern blot analysis, and 10 μ g of each total bacterial protein was used for Western blot analysis. Lanes A1 and C1, AH2 (wild type); lanes A2 and C2, AH2552 (RpfA⁻ [GacS⁻] of AH2); lanes B1 and D1, AH2 carrying the cloning vector pLARF5; lanes B2 and D2, AH2 carrying the GacS⁺ plasmid pAKC1052; lanes B3 and D3, AH2552 carrying pLARF5; lanes B4 and D4, AH2552 carrying pAKC1052; lane E1, AH2; lane E2, AH2552; lane E3, AH2 carrying pLARF5; lane E4, AH2 carrying pAKC1052; lane E5, AH2552 carrying pLARF5; lane E6, AH2552 carrying pAKC1052.

RNA affects exoprotein production and other traits by neutralizing the effects of RsmA. On the basis of these findings, we predict that GacA positively affects *rsmB* transcription and high levels of *rsmB* RNA renders RsmA inactive, thereby causing overproduction of exoproteins. According to this hypothesis, *gacA*⁺ DNA would have relatively little effect in RsmA⁺ bacteria. To test this possibility, we transferred the GacA⁺ plasmid or the cloning vector into AC5071 and its RsmA⁺ parent Ecc71 and culture supernatants were assayed for enzymatic activities. The data for Pel levels (Table 1) show that GacA stimulates enzyme production in the RsmA⁺ bacteria by approximately 15-fold and in the RsmA[−] bacteria by approximately threefold. We extended these observations by examining the effect of GacA in a RsmA[−]RsmB[−]RsmC[−] mutant AC5055 because, despite extensive attempts, we were unable to make a *rsmB*[−] single mutant. Note that RsmC controls exoenzyme production by positively regulating *rsmA* transcription and negatively regulating *rsmB* transcription (Cui et al. 1999). The results (Table 1 and Fig. 1A, columns 5 to 8; B, lanes 5 to 8; and D, columns 5 to 8) showed that multiple copies of *gacA* did not affect exoenzyme production nor the levels of *pel-1*, *peh-1*, *celV*, and *hrpN*_{Ecc} transcripts and

Fig. 3. A, β -galactosidase assays of *Erwinia carotovora* subsp. *carotovora* strain AC5006 and its GacA[−] mutant AC5057 carrying pAKC1018 (*rsmB*_{Ecc}-*lacZ*), pAKC1047 (*rsmB*_{Ea}-*lacZ* fusion), pAKC1048 (*rsmB*_{Ehg}-*lacZ* fusion), or the cloning vector PMP220. B, β -galactosidase assays of *Pseudomonas syringae* pv. *syringae* strain B728a, its GacA[−] mutant BGACX and its GacS[−] mutant NPS3136 carrying pAKC1047, pAKC1048, or the cloning vector PMP220. Bacterial constructs were grown in minimal salts medium plus sucrose and tetracycline at 28°C to a Klett value of approximately 200. Bacterial cultures were harvested and used for assays.

Harpin_{Ecc} in the RsmA[−]RsmC[−] and the RsmA[−]RsmB[−]RsmC[−] mutants. We previously documented that multiple copies of *rsmB* DNA does not affect these phenotypes in the RsmA[−]RsmC[−] mutant (Cui et al. 1999). These findings provide very strong evidence that GacA affects the expression of *rsmB* and the GacA effect in *E. carotovora* subsp. *carotovora* is mediated mostly via the RsmA-*rsmB* posttranscriptional regulatory system.

Occurrence of *gacA* and *gacS* homologs in *Erwinia* species.

The positive effects of GacS-GacA on transcription of *rsmB* genes of *E. amylovora* and *E. herbicola* pv. *gypsophylae* suggest that this two-component system occurs in these non-soft-rotting bacteria as well. To test this idea, we conducted Southern blot hybridization with *gacA* and *gacS* DNA segments of Ecc71. The results (Fig. 5) indicate that homologs of these genes occur in soft-rotting bacteria in *E. carotovora* subsp. *carotovora*, *E. carotovora* atroseptica, *E. carotovora* betavascularum, and *E. chrysanthemi* as well as in the non-soft-rotting bacteria in *E. amylovora*, *E. rhapontici*, *E. herbicola*, *E. stewartii*, and *E. herbicola* pv. *gypsophylae*. Thus, it is evident that the sequences corresponding to this two-component regulatory system have been conserved in all of these plant-pathogenic and plant-associated bacteria.

DISCUSSION

In this report, we have shown that the two-component system comprising GacS and GacA controls extracellular protein production by modulating the levels of *rsmB* RNA. This conclusion is supported by the following findings: i) the occurrence of low levels of *rsmB* RNA in a GacS[−] mutant and a GacA[−] mutant; ii) restoration of *rsmB* transcript levels in these mutants by the *gacS*⁺ DNA or *gacA*⁺ DNA; iii) the positive effect of the dosage of *gacA*⁺ DNA or *gacS*⁺ DNA on *rsmB* RNA levels; and iv) high levels of expression of *rsmB*-*lacZ* transcriptional fusions in *E. carotovora* subsp. *carotovora* strain AC5006 or *P. syringae* pv. *syringae* strain B728a, yet much reduced levels of expression of these fusions in *E. carotovora* subsp. *carotovora* GacA[−] strain AC5057 and *P. syringae* pv. *syringae* GacS[−] mutant NPS3136 and GacA[−] mutant BGACX. Additional evidence comes from the observation that GacA stimulates *rsmB* transcription but does not affect other regulators such as HexA (Harris et al. 1998; Mukherjee et al. 2000), KdgR (Liu et al. 1999), RsmA (Chatterjee et al. 1995; Cui et al. 1995), and RsmC (Cui et al. 1999). Moreover, the GacA effect manifests itself in RsmA⁺,

Fig. 4. Effect of *gacA* and *gacS* on levels of A and B, *rsmA* transcripts and C, RsmA protein. Bacterial constructs were grown in minimal salts medium plus sucrose to a Klett value of approximately 150 for total RNA isolation and to a Klett value of approximately 200 for total bacterial protein extraction. Lane A1, Ecc71; lane A2, AC5056; lane B1, AH2; lane B2, AH2552; lane C1, Ecc71; lane C2, AC5056; lane C3, AH2; lane C4, AH2552. Lanes A1, A2, B1 and B2 contained 10 μ g of total RNA and lanes C1-C4 contained 20 μ g of total bacterial protein.

RsmC⁺, and *rsmB*⁺ strains, but not in bacteria that are deficient in RsmA, RsmC, and *rsmB* RNA. These observations strongly suggest that GacS–GacA act via the RsmA–*rsmB* pathway to control exoprotein production. Note that other transcriptional factors such as HexA, RsmC, and KdgR, which are known to affect exoprotein production in *E. carotovora* subsp. *carotovora*, also affect the RsmA–*rsmB* posttranscriptional regulatory system. These findings collectively support the notion that RsmA and *rsmB* RNA are the central regulators, whereas other regulators and signals are channeled via this system to rigorously control extracellular enzyme production.

The effects of GacS–GacA on harpin production in *E. carotovora* subsp. *carotovora* contrasts with the lack of an effect of this regulatory system on the induction of the HR or the expression of the *hrp* genes in *P. syringae* pv. *syringae* (Willis et al. 1990). In a series of studies, we documented that the transcription of *hrpN* and other *hrp* genes (Chatterjee et al. 2000) is controlled rigorously by the RsmA–*rsmB* pathway. Because GacS–GacA act via this pathway (see above), the results with *E. carotovora* subsp. *carotovora* *hrp* genes were expected.

Although there is now convincing evidence for the activation of *rsmB* transcription by GacS–GacA, we do not know for certain if *rsmB* is the primary target of these regulators. This is, however, suggested by the expression of *rsmB* in heterologous systems. For example, GacA of Ecc71 stimulated *csrB* transcript levels in *E. coli*. Likewise, *lacZ* fusions of *Erwinia* spp. *rsmB* genes are expressed in a *P. syringae* pv. *syringae* strain. We have not yet ruled out the possibility, however, that these bacteria possess another regulator of *rsmB*, which is the primary target for GacA-mediated activation. We initiated genetic studies to rule out this possibility. In addition, we are in the process of purifying GacA to test its interaction with the *rsmB* DNA and its ability to stimulate in vitro transcription of *rsmB*.

The regulation of many different traits by GacS–GacA in different bacteria (Corbell and Loper 1995; Dziejamn and Mekalanos 1996; Eriksson et al. 1998; Frederick et al. 1997; Lam et al. 1994; Laville et al. 1992; Whistler et al. 1998) raises the following issues. Is the GacA effect mediated via *rsmB* or its functional homologs in each of these instances? If *rsmB* is the primary target in these bacteria, how are the different phenotypes affected? To resolve these issues, we began

searching for *rsmA*- and *rsmB*-like genes in various plant-pathogenic pseudomonads. We know that homologs of *rsmA* and *rsmB* occur in most, if not all, enterobacteria (Cui et al. 1995; Dumenyo et al. 1999; Murata et al. 1994; White et al. 1996). Moreover, *rsmA* homologs have been cloned from *Pseudomonas aeruginosa* (GenBank accession no. AF061757) and *P. fluorescens* (Blumer et al. 1999). In fact, a BLAST search of the whole genome sequence data revealed that *rsmA* homologs are present in *Bacillus subtilis*, *Campylobacter jejuni*, *Clostridium acetabutylicum*, *Clostridium difficile*, *Haemophilus influenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Pasturella multocida*, *Shewanella putrefaciens*, *Thermotoga maritima*, *Treponema pallidum*, and *Vibrio cholerae*. A recent paper by Aarons et al. (2000) described the effect of GacA–GacS of *P. fluorescens* on the expression of *prfB* RNA (a *rsmB* homolog) and consequent secondary metabolite production. Thus, we are fairly certain that *rsmA*- and *rsmB*-like genes will be found in *P. syringae* pathovars that cause diseases in many plant species as well as in pathovars where GacA–GacS control various phenotypes. Once we have obtained *rsmA*- and *rsmB*-like genes from these bacteria, it is a relatively straightforward process to test the effects of GacS–GacA on these genes and to assess the phenotypic changes that result from the inactivation of *rsmA* and *rsmB* homologs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids are described in Table 2. The strains were maintained on Luria-Bertani (LB) agar or LB agar containing appropriate antibiotics.

The compositions of LB medium, minimal salts medium, nutrient gelatin agar, and polygalacturonate–yeast extract agar have been described (Chatterjee et al. 1995; Murata et al. 1991). When required, antibiotics and drugs were supplemented with, per ml, 100 µg of ampicillin, 50 µg of kanamycin, 50 µg of spectinomycin, 20 µg of Gentamicin (Gm), and 10 µg of tetracycline. Media were solidified by the addition of 1.5% agar.

The compositions of agarose media for semiquantitative assays of enzymatic activities were described in Chatterjee et al. (1995).

Fig. 5. Southern blot hybridization of *EcoRI*-digested chromosomal DNAs of *Erwinia* spp. strains with **A**, *gacA* and **B**, *gacS*. Lanes 1–5, *Erwinia carotovora* subsp. *carotovora* strains Ecc71, Ecc193, SCRI193, SCC3193, and AH2, respectively; lane 6, *E. carotovora* subsp. *atroseptica* strain Eca12; lane 7, *E. carotovora* subsp. *betavascularum* strain Ecb11129; lane 8, *Erwinia chrysanthemi* strain EC16; lane 9, *Erwinia amylovora* strain E9; lane 10, *Erwinia rhapontici* strain Er1; lane 11, *Erwinia herbicola* strain EH105; lane 12, *E. herbicola* pv. *gypsophilae* strain PD713; lane 13, *Erwinia stewartii* strain DC283.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
Ecc71	Wild type	Zink et al. 1984
AC5006	Lac ⁻ derivative of Ecc71	Murata et al. 1991
AC5071	RsmA ⁻ derivative of Ecc71, Km ^r	Mukherjee et al. 1997
AC5054	RsmC ⁻ derivative of AC5071 Km ^r , Sp ^r	Cui et al. 1999
AC5055	RsmB ⁻ derivative of AC5054 Cm ^r , Km ^r , Sp ^r	Liu et al., <i>unpublished</i>
AC5056	GacA ⁻ derivative of Ecc71, Gm ^r	This study
AC5057	GacA ⁻ derivative of AC5006, Gm ^r	This study
Ecc193	Wild type	Zink et al. 1984
SCRI193	Wild type	Salmond et al. 1986
SCC3193	Wild type	Pirhonen et al. 1993
AH2	Wild type	Frederick et al. 1997
AH2552	RpfA ⁻ (GacS ⁻) derivative of AH2	Frederick et al. 1997
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>		
Eca12	Wild type	Zink et al. 1984
<i>Erwinia carotovora</i> subsp. <i>betavascularum</i>		
Ecb11129	Wild type	J.E. Loper, <i>unpublished</i>
<i>Erwinia chrysanthemi</i>		
EC16	Wild type	Chatterjee et al. 1979
<i>Erwinia amylovora</i>		
E9	Wild type	Politis and Goodman 1980
<i>Erwinia herbicola</i>		
EH105	Wild type	Cui et al. 1995
<i>Erwinia herbicola</i> pv. <i>gypsophillae</i>		
PD713	Wild type	Lichter et al. 1995
<i>Erwinia stewartii</i>		
DC283	Wild type	D. Coplin, <i>unpublished</i>
<i>Erwinia rhapontici</i>		
Er1	Wild type	McEvoy et al. 1990
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B728a	Wild type	Willis et al. 1990
BGACX	GacA ⁻ derivative of 0728a, Km ^r	Rich et al. 1994
NPS3136	GacS ⁻ derivative of 0728a, Km ^r	Willis et al. 1990
<i>Escherichia coli</i>		
DH5 α	ϕ 80lacZ Δ M15 Δ (lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1	Gibco BRL, Gaithersburg, MD, U.S.A.
MC4100	araD139 Δ (lacIPOZYA)U169 recA1 thi-1 Str ^r	Liu et al. 1994
pBluescript-SK(+)	Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pCL1920	Sp ^r , Sm ^r	Lerner and Inouye 1990
p34S-Gm	Gm ^r , source of Gm ^r cassette	Dennis and Zylstra 1998
pLARF5	Tc ^r	Keen et al. 1988
pMP220	Tc ^r , promoter-probe vector	Spaink et al. 1987
pRK415	Tc ^r	Keen et al. 1988
pAKC781	Ap ^r , <i>peh-1</i> ⁺	Liu et al. 1994
pAKC783	Ap ^r , <i>pel-1</i> ⁺	Liu et al. 1994
pAKC882	Ap ^r , pT7- <i>rsmA</i>	Mukherjee et al. 1996
pAKC924	Ap ^r , <i>hrpN</i> _{Ecc} in pBluescriptSK(+)	Cui et al. 1996
pAKC975	Sp ^r , 2.0 kb of RsmC ⁺ DNA in pCL1920	Cui et al. 1999
pAKC1004	Sp ^r , 316 bp <i>rsmB</i> DNA in pCL1920	Liu et al. 1998
pAKC1018	Tc ^r , <i>rsmB</i> _{Ecc} - <i>lacZ</i> in pMP220	Liu et al. 1998
pAKC1025	Tc ^r , <i>kdgR</i> _{Ecc} ⁺ DNA in pRK415	Liu et al. 1999
pAKC1034	Ap ^r , 200 bp DNA fragment of <i>celV</i> from Ecc71 in pGEM-T Easy	Liu et al. 1999
pTB2	Ap ^r , 4.3 kb of RpoS ⁺ DNA in pGem3Z	Calcutt et al. 1998
pAKC1047	Tc ^r , <i>rsmB</i> _{Ea} - <i>lacZ</i> , 1.4-kb <i>rsmB</i> _{Ea} DNA in pMP220	Lab collection
pAKC1048	Tc ^r , <i>rsmB</i> _{Ehg} - <i>lacZ</i> , 1.0-kb <i>rsmB</i> _{Ehg} DNA in pMP220	Lab collection
pAKC1051	Tc ^r , pLARF5 containing <i>gacA</i> from genomic library of Ecc71	This work
pAKC1052	Tc ^r , pLARF5 containing <i>gacS</i> from genomic library of Ecc71	This work
pAKC1053	Ap ^r , GacA ⁺ , 1.0-kb polymerase chain reaction (PCR) product of <i>gacA</i> from Ecc71 in pGEM-T Easy	This work
pAKC1054	Ap ^r , 217-bp PCR product of <i>gacS</i> from Ecc71 in pGEM-T Easy	This work
pAKC1055	Tc ^r , GacA ⁺ , 2.1-Kb <i>Bam</i> HI- <i>Hind</i> III fragment of pAKC1051 in pRK415	This work
PAKC1056	Ap ^r , 1.2-kb PCR product of <i>gacS</i> from Ecc71 in pGEM-T Easy	This work
pAKC1057	Gm ^r , Tc ^r , Gm ^r cassette inserted in <i>Eco</i> RV site of pAKC1055	This work

Preparation of samples

for enzyme assays and assay conditions.

The procedures described by Murata et al. (1991) were followed for the preparation of enzyme samples for quantitative extracellular Pel enzymatic assays. The semiquantitative agarose plate assays for extracellular Peh, Prt, and Cel were performed according to Chatterjee et al. (1995). All assays were repeated at least three times with comparable results.

Construction

of *E. carotovora* subsp. *carotovora* GacA⁻ mutants.

To inactivate *gacA*, the Gm^r cassette was inserted at the *EcoRV* site of *gacA* DNA fragment in pAKC1055 to produce pAKC1057. pAKC1057 was transferred into Ecc71 or AC5006 with the helper plasmid, pRK2013. Transconjugants were selected on minimal salts agar containing sucrose and Gm. Gm^r and Tc^r isolates were selected for further studies. The marker exchanges were confirmed by Southern blot analysis.

DNA techniques.

Standard procedures were used in the isolation of plasmid and chromosomal DNAs, electroporation, restriction endonuclease digests, gel electrophoresis, and DNA ligation (Sambrook et al. 1989). Southern blot analysis was carried out as described by Cui et al. (1995). The 1.0-kb *EcoRI* fragment of pAKC1053 and 1.2-kb *EcoRI* fragment of pAKC1056 were used as *gacA* and *gacS* probes, respectively. PCR was performed as described by Liu et al. (1998). The primers used to amplify *gacA* and *gacS* were PgacA1, 5'-GTGATCACGATG CGCCCTCAGGTT-3' (responding to nucleotides (nt) -187 to -163 from translational start site of *expA*); PgacA2, 5'-CTG CGGAAGTAGCTGGCAAGTCGT-3' (responding to 115 to 139 nt downstream of *expA*); PgacS1, 5'-CAGTCTTCGTGC ACGGATGTTGAT-3' (responding to 12 to 36 nt from translational start site of *expS*); and PgacS2, 5'-CGTCATTCTGAA TACTGAGCGTCA-3' (responding to 1,148 to 1,172 nt from the translational start site of *expS*). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, WI, U.S.A.). The Primer-a-Gene DNA labeling system from Promega Biotec was used to label DNA. Nucleotide sequences were determined at the DNA Core Facility of the University of Missouri-Columbia. The DNA and protein sequences were analyzed by BLAST.

RNA isolation and Northern blot analyses.

Bacterial cultures were grown at 28°C in minimal salts medium supplemented with sucrose (0.5% wt/vol) and appropriate antibiotics. Cells were collected when cultures had reached a value of approximately 150 Klett units. The procedures for RNA isolation and Northern blot analysis have been described (Liu et al. 1993). The probes used in this work were the 183-bp *NdeI*-*SalI* fragment of *rsmA* from pAKC882 (Muhkerjee et al. 1996), the 321-bp *BamHI*-*HindIII* fragment of *rsmB* from pAKC1004 (Liu et al. 1998), 304-bp *EcoRV*-*HindIII* fragment of *rsmC* from pAKC975 (Cui et al. 1999), 314-bp *EcoRV*-*KpnI* fragment of *pel-1* from pAKC783 (Liu et al. 1994), 743-bp *HindIII* fragment of *peh-1* from pAKC781 (Liu et al. 1994), 200-bp *EcoRI* fragment of *celV* from pAKC1034 (Liu et al. 1999), 779-bp *EcoRV*-*SmaI* fragment of *hrpN_{Ecc}* from pAKC924 (Cui et al. 1996), 527-bp *BstEII*-

EcoRI fragment of *kdgR* from pAKC1025 (Liu et al. 1999), and 875-bp *HpaI*-*MluI* fragment of *rpoS* from pTB2 (Calcutt et al. 1998).

Western blot analysis.

Bacterial constructs were grown at 28°C in minimal salts medium plus sucrose (0.5% wt/vol), with or without tetracycline, to a Klett value of approximately 200. Total bacterial protein was precipitated with trichloroacetic acid at a final concentration of 10% (vol/vol) and resuspended in 1× sodium dodecyl sulfate loading buffer (Sambrook et al. 1989). The concentrations were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.), according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was performed as described by Muhkerjee et al. (1997). The antibodies raised against the Harpin from *E. chrysanthemi* (Bauer et al. 1995) was used as the probe for Harpin_{Ecc}. The anti-RsmA antiserum described by Cui et al. (1999) was used as the probe for RsmA.

β-galactosidase assay.

E. carotovora subsp. *carotovora* strain AC5006 and its GacA⁻ mutant AC5057 carrying pAKC1018 (*rsmB_{Ecc}*-*lacZ*), pAKC1047 (*rsmB_{Ea}*-*lacZ*), pAKC1048 (*rsmB_{Ehg}*-*lacZ*), or the cloning vector (pMP220) and *P. syringae* pv. *syringae* strain B728a, its GacA⁻ derivative BGACX and its GacS⁻ derivative NPS3136 carrying pAKC1047, pAKC1048, or vector pMP220 were grown in minimal salts medium plus sucrose (0.5% wt/vol) and tetracycline to a Klett value of approximately 200 and harvested. The β-galactosidase assays were carried out according to Miller (1972).

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