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GacS and GacA, members of a two component system, positively control virulence factors of the fire-blight pathogen, *Erwinia amylovora* by modulating the levels of *rsmB* RNA*

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The two-component regulatory system comprising GacA and GacS proteins occurs in many prokaryotes. To elucidate their regulatory functions in *E. amylovora* (Ea), we obtained GacA$^+$ and GacS$^+$ cosmids from a gene library of the strain E9 and constructed Ea GacA$^-$ and GacS$^-$ mutants by marker exchange. The GacA$^-$ and GacS$^-$ mutants, compared to wild type strain E9, produced reduced levels of exopolysaccharide (EPS) and siderophores and exhibited reduced swarming motility; these phenotypes were restored to the wild type level by the GacA$^+$ and GacS$^+$ consmids in the cognate mutants. The mutants required higher cell density than the GacA$^+$ parent to cause the fire blight disease in apple shoots and to elicit the hypersensitive response (HR) in tobacco leaves. Northern blot analysis showed that GacA$^-$ and GacS$^-$ mutants, compared to E9, produced much reduced levels of transcripts of *hrpL,* the gene for an alternate sigma factor, transcripts of *hrpN* and *dspB* genes for effectors required for plant interaction and transcripts of *dfoA* (gene for the siderophore, desferrioxamine synthesis). Several lines of evidence establish that the GacS/GacA effects were channeled via *rsmB,* which specifies a non-coding regulatory RNA. (1) The levels of *rsmB* RNA were barely detectable in the mutants. (2) The expression of an *rsmB-lacZ* transcriptional fusion was severely reduced in the absence of GacS or GacA. (3) The introduction of *gacA*$^+$ DNA or *gacS*$^+$ DNA into the cognate mutants restored *rsmB* RNA production and *rsmB-lacZ* expression. (4) Increasing the dosage of *rsmB* DNA reversed the phenotypic changes resulting from deficiency fo GacA or GacS. (5) The putative GacA binding sequences are present within the promoter region of *rsmB.*

Key words: Pathogenicity factors, host defence, two component regulatory system, post transcriptional regulatory factors

INTRODUCTION

Erwinia amylovora (Ea) is a Gram-negative bacterium which causes fire blight disease on several Rosaceous plants such as apples, pears, quince, raspberry and other ornamentals (Oh and Beer, 2005). In non-host plants, such as tobacco and *Arabidopsis,* Ea causes hypersensitive-like reaction (Degrave *et al.,* 2008; Boureau *et al.;* 2006). Ea forms short rod-shaped cells with peritrichous flagella, responsible for bacterial movement inside the plant and in the environment away from plant. Both genetic and physiological data have established that Ea produces multiple factors including exopolysaccharide (EPS) (Ayers *et al.,* 1979; Bugert and Geider, 1995), siderophores (Expert, 1999; Dellagi *et al.,* 1998) and proteins (effectors) secreted by the Type III secretion system (Oh and Beer, 2005; Bocsanczy *et al.,* 2008) that are required for disease development.

Ea produces the heteropolysaccharide, amylovoran and the homopolymer, levan. The synthesis of amylovoran is critical for virulence of Ea since the strains which are unable to produce it fail to invade the host. Amylovran biosynthesis requires as many as a dozen genes clustered together into what is known as the *ams* operon (Bugert and Geider, 1995). These genes are tightly regulated by *rcsA, rcsB,* and *rcsC* (Bereswill and Geider, 1997; Coleman *et al.,* 1990; Keim *et al.,*1997). A previous study has also showed that the post-transcriptional

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regulatory system comprising RsmA-*rsmB* RNA pair regulates EPS production in *E. amylovora* (Ma *et al.,* 2001). It is believed that EPS protects bacteria from host defenses, allows bacteria to move through the cortex, and prevents water flow in vascular tissues, thereby triggering water stress and wilting.

E. amylovora produces siderophores which function as chelators and transporters of iron (III) (Expert, 1999). These small compounds play an important role under iron limited conditions including those prevailing in host tissues. Genetic evidence has disclosed that *dfoA,* responsible for DFO (desferriozamine) biosynthesis and *foxR,* ferrioxamine receptor gene contribute to symptom production in apple flowers (Kachadourian *et al.,* 1996; Dellagi *et al.,* 1998, Dellagi *et al.,* 1999).

Like many Gram negative becterial pathogens, Ea possesses cluster of genes that specify the type III (Hrp) secretion pathway and the proteins (effectors) secreted by this pathway. In Ea, as in *Pseudomonas syringae,* the ability to cause the HR as well as the disease, depends on *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes. Ea produces harpin, HrpA pilus, and Avr-like proteins (Bocsanczy *et al.,* 2008 and references cited therein). Harpins and HrpA pilin are encoded by *hrpN* and *hrpA* genes, respectively, and the Avr-like proteins are encoded by *dsp* genes. The phytotoxicity of Harpin of *E. amylovora* and genetic evidence for its role in virulence has been well-established (Wei *et. al.,* 1992). HrpL, the ECF subfamily of alternate sigma factors, regulates the expression of *hrp* and *dsp* genes (Wei and Beer, 1995; Bocsanczy *et. al.,* 2008).

Multi-factorial pathogenicity demands that pathogenicity/virulence determinants must be produced in an orderly manner.Pathogens use global as well as gene specific regulators that ensure their production in a temporal sequence. While several gene specific regulators have been identified in Ea, relatively less is known of the global regulators. One exception is the post-transcriptional regulators comprising of RsmA, an RNA-binding protein and rsmB RNA that bind RsmA. We have shown that these regulators control various pathogenicity determinants in Ea (Ma *et al.,* 2001).

Given the widespread occurrence of a two-component regulatory system comprising the sensor kinase, GacS, and the response regulator, GacA, in the prokaryotic world (Heeb and Haas, 2001; Lapouge *et al.,* 2008), we considered the possibility that these regulators may also control pathogenicity factors in Ea. In this regard we should note that the GacS/GacA system controls production of bacterial metabolites required for pathogenicity to plants and animals, biocontrol of plant diseases, ecological fitness or tolerance to stress (for relevant references see Lapouge *et al.,* 2008). A current model proposes that GacS senses an unknown signal(s) and stimulates GacA, transcriptional regulator, through a phospho-relay mechanism, which then triggers the transcription of the targeted genes. GacS, the specific sensor kinase, is capable of autophosphorylation, and it prossesses a receiver domain and an output domain. The periplasmic domain of GacS is fairly diverse among bacterial strains, which implies that the signals recognized by GacS are diverse as well. Based upon evidence with other bacteria (cited in Lapouge *et al.,* 2008) it is now possible to speculate on the mechanism underlying the activation of the target genes by GacA. Phosphorylated GacA (GacA-P) is presumed to bind a conserved 18 base sequence [TGTAAGN6 CTTACA, where N is any nucleotide], dubbed as the GacA box. These sequence occur in upstream regions of *rsmB,* non-coding sRNA genes of various bacteria including the soft-rot pathogen, *E. carotovora* ssp. *carotovora.* In this bacterium, GacS/A activates the expression of *rsmB* RNA, which in turn stimulates the expression of an assortment of genes (Eriksson *et al.,* 1998; Frederick *et al.,* 1997; Cui *et al.,* 2001).

A previous report has provided evidence for the presence of *gacS* and *gacA* DNA sequences in Ea (Cui *et al.,* 2001). We have extended that observation and report here the regulatory effects of the GacS- GacA system in *E. amylovora.* Our data show that compared to the wild type strain E9, its GacA$^-$ and GacS$^-$ mutants produce significantly low levels of EPS and siderophores. Moreover, these mutants exhibit severely reduced swarming motility and require at least ten times higher cell density to cause fire blight in apples and the HR in tobacco leaves. The transcript levels of several *hrp* genes, *dspA, dfoA* and *rsmB* RNA are much lower in mutants compared to the levels in E9. Our findings establish that this two component system, as in many other bacteria, functions as a global regulator in Ea, and that the GacS-GacA effects are channeled via its regulatory effect on *rsmB.*

While this manuscript was in preparation, Zhao *et al.* (2009) have published a report describing a systems level analysis of two component signal transduction systems of *E. amylovora*. They have reported that a two component system, analogous to the GacS and GacA system (their designations GrrS and GrrA, respectivety) negatively regulates amylovoran production and swarming motility. Moreover, mutants deficient in GrrA or GrrS are not affected in virulence. These findings contrast with positive effects of GacS and GacA on amylovoran and siderophore production, swarming motility and virulence of *E. amylovora*. Thus, although GacS and GacA share significant homology with GrrS and GrrA, respectively, they are functionally quite different. We show here that GacSA positively regulates rsmB RNA production, required for the neutralization of the negative effects of RsmA. These findings explain the basis for positive regulatory effects of GacSA in *E. amylovora* and demonstrate functional similarities with GacSA of various *Pseudomonas* and other *Erwinia* species. A simplest and most likely explanation for these seemingly contradictory results are that the two component system reported by Zhao *et al.*, (2009) is quite different from the GacSA system described here.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are described in Table 1. The strains carrying drug markers were maintained on Luria-Bertani (LB) agar containing appropriate antibiotics. The wild-type strains were maintained on LB agar. The composition of LB medium, minimal salts medium, King's B (KB) medium, nutrient gelatin agar, and polygalacturonate-yeast extract agar (PYA) were described previously (Chatterjee *et al.*, 2003; Murata *el al.*, 1991). When required, antibiotics were supplemented as follows (in micrograms per milliliter): kanamycin, 50; spectinomycin, 50; tetracycline, 10. Media were solidified by the addition of 1.5% (wt/vol) agar.

EPS production and motility assays

Cultures of *E. amylovora* wild type and the other derivative strains were patched or streaked on KB agar medium for EPS detection and stab inoculated into KB soft agar (0.4%, wt/vol) with a needle for examination of motility. Plates were incubated at 28°C for 24 hrs. Motility and EPS production were visually examined.

Table 1 : Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Source/Reference
Strains		
Erwinia carotovora ssp. *carotovora*		
Ecc71	wild-type	Zinke *et al.*, 1984
AC5057	Gm^r, $GacA^-$ derivative of Ecc71	Cui *et al.*, 2001
AH2552	$GacS^-$ derivative of AH2	Frederic *et al.*, 1997
Erwinia amylovora		
E9	Wild type	Politis and Goodman, 1980
AC5200	Km^r, $GacA^-$ derivative of E9	This study
AC5201	Km^r, $GacS^-$ derivative of E9	This study
Escherichia coli		
DH5α	ϕ80*lacZ*Δ*M15* Δ*(lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
Plasmids		
pLARF5	Tc^r	Keen *et al.* 1988
pCL1920	Sp^r, Sm^r	Lerner and Inouye 1990
pMP220	Tc^r, promoter-probe vector	Spaink *et al.* 1987
pAKC1043	Sp^r, *plac-rsmB* in pCL1920	Ma *et al.*, 2001
pAkC1047	Tc^r, *rsmB-lacZ* in pMP220	Cui *et al.*, 2001
pAKC2000	Tc^r, *gacA*$^+$ in pLARF5	This study
pAKC2001	Tc^r, *gacS*$^+$ in pLARF5	This study

Siderophores production

For the semi-quantitative plate assay, we used Chrome Azurol S (CAS) agar medium. Cultures of *E. amylovora* strains were grown in KB medium to a value of approximately 100 Klett units and used for assays. Samples were spotted on plates and after 16 to 18 hrs. the orange halo was observed.

Elicitation of HR

E. amylovora strains were grown on LB agar overnight at 28°C. Cells were suspended in water and used for the HR as previously described (Mukherjee *et al.,* 1997). Bacterial suspension was infiltrated into the petioles. Pictures were taken after 18 hrs.

Pathogenicity assay on apple shoots

Pathogenicity assay on apple shoots were carried out essentially as previously described (Mukherjee *et al.,* 1996). Cell suspensions to a concentration of approximately 2×10^{10} cfu/ml of *E. amylovora* strains were applied to the cut surface of each petiole. Inoculated plants were incubated at 28°C with a 14-h light/10-h dark regime until disease symptoms appeared around 4-5 days.

DNA technique

Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformations, and DNA ligation (Sambrook *et al.,* 1989). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, WI). The Primer-a-Gene DNA labeling system of Promega Biotec was used for labeling 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 hybridization

Bacterial cultures were grown at 28°C in KB medium to a Klett value of 150 and the total RNA was extracted and used for expression of *rsmB* and *dfoA* genes. For *hrp* genes, when cultures reached a value of approximately 100 klett unit in KB medium cells were collected, washed with *hrp* inducing medium (Huang *et al.,* 1995), and resuspended in same amount of hrp inducing medium. The cells were incubated at 28°C for additional 2 hrs before RNA isolation. The procedures for RNA isolation and Northern blot analysis have been described (Liu *et al.* 1994).

Construction of E. amylovora GacA⁻ and GacS⁻ mutants

To construct GacA and GacS derivative of E9, we inactivated the *gacA* and *gacS* loci in pAKC2000 and pAKC2001 cosmids by transposon mutagenesis using Tn5Km cassette. We mutagenized pAKC2000 and pAKC2001 with Tn5 cassette, selected for Km and Tc resistant transconjugants of *E.coli* DH5α on LB Km and Tc agar medium. Plasmid DNAs from the drug-resistant colonies were isolated and introduced into Ecc71 GacA⁻ of AHH2 as well as in Ea strain E9 for phenotype restoration. Those colonies that did not produce exoenzymes were presumed to carry inactivated genes. The insertions of the transposon within *gacA* or *gacS* loci of pAKC2000 or pAKC2001 were confirmed by isolating plasmid DNAs and performing Sourthern blot hybridization with $gacA_{Ecc}$ or $gacA_{Ecc}$ as probe. These mutagenized cosmids did not restore the phenotypes in Ecc71 GacA⁻ and AHH2. Moreover, they did not hyper stimulate production or swarming motility in Ea strain E9 whereas non-mutagenized cosmids (pAKC2000 and pAKC2001) stimulated EPS production and swarming motility.

We constructed E9 GacA⁻ and GacS⁻ mutants by marker exchange. We introduced these mutagenized cosmids into E9 wild type and obtained colonies that are Km resistant and Tc sensitive. One out of about 2,000 colonies for GacA and four out of 1,000 colonies were obtained for GacS. Chromosomal DNA of these mutant colonies were isolated and disruption of the cognate genes were confirmed by Southem blot analysis with $gacA_{Ecc71}$, $gacS_{Ecc71}$, and Tn5 probes. For subsequent studies, we selected AC5200 as the representative of GacA⁻ mutants and AC5201 as GacS⁻ mutants.

RESULTS

Characterization of gacA and gacS genes of E. amylovoroa strain E9

Despite the evidence for the presence of *gacS* and *gacA* homologs in *E. amylovora* strain E9 (Cui *et*

al., 2001), the genes were not structurally or functionally characterized. For such characterizations, we isolated these genes from a genomic library of *E. amylovora* strain E9 in the cosmid vector, pLAFR5. The library was mobilized into a GacA$^-$ mutant (AC5057) of *E. carotovora* ssp. *carotovora* (Ecc) strain of Ecc71 or a Ecc GacS$^-$ strain AH2552 and the transconjugants were screened for extracellular pectinase production. Since pectinase production in AC5057 or AH2552 is severely reduced (Cui *et al.,* 2001), any transconjugant which restored the production of pectinase was presumed to carry *gacA* gene, *gacS* gene or another regulator gene, such as *rsmB,* that could compensate for GacA deficiency. After screening ca. 1,000 transconjugants we obtained several pectinase producing clones. Following analysis of PCR products and Southern results, we concluded that the pectinase producing transconjugants carried *gacS, gacA* or *rsmB* genes. For further analysis we used pAKC2000 carrying *gacA* and pAKC2001 possessing *gacS.* Ecc GacA$^-$ strain AC5057 carrying pAKC2000 and Ecc GacS$^-$ strain AH2552 carrying pAKC2001 also restored tissue meceration on celery similar to their wild type parents, whereas the mutants carrying the vector, pLAFR5 did not (data not shown). These observations established that the Ea genes are fully functional in the soft-rotting bacterium.

The sequence alignment results revealed that the deduced amino acid sequences of E9 GacA and GacS have strong homology with previously reported GacA and GacS homologs of various enterobacterial species. The per cent identities of E9 GacA and GacS with the cognate proteins, shown parenthetically in that order, are as follows; *Erwinia pyrifoliae* (accession number YP–002649213; 98% identical and accession number YP–0026449825.1; 96% identical), *Erwinia tasmaniensis* strain Et1/99 (accession number YP–001908002.1; 94% identical and accession number YP–001908642.1; 91% identical); *Pantoea* sp. At-9b (accession number ZP– 05731299.1; 86% identical and accession number ZP–05732155.1; 71% identical); *E. coli* (accession number YP–003234908.1; 93% identical and number YP–003035194.1; 66% identical); and *Erwinia carotovora* ssp. *atrosepticum* strain SCRI1043 (accession number YP–050973.1; 76% identical and accession number YP–051659.1; 62% identical).

GacA and GacS positively control EPS production, swarming motilty, and siderophore production

EPS production, swarming motility, and siderophores production are required for pathogenicity of *E. amylovora.* To determine the effects of GacA and GacS of Ea, on EPS production E9 GacA$^-$ mutant (AC5200) and GacS$^-$ mutant (AC5201) and the wild type parent were grown in King's B (KB) agar medium. Visual examination of such assays revealed that compared to the wild type strain E9,both GacA$^-$ and GacS$^-$ mutants produced significantly less EPS (Fig. 1A). For swarming motility, bacteria were stab-inoculated on KB soft agar (0.4%, wt/vol) and visually examined. Similar to EPS production, both mutants severely reduced their swarming motility compared to the wild type (Fig. 1B).

Siderophore production was assayed on Chrome Azurol S (CAS) agar medium. The results (Fig. 1) showed that E9 (C2) produced higher level of siderophore than both GacA$^-$ (C1) and GacS$^-$ (C3) mutants. We extracted total RNA and performed Northern blot analysis to determine the expression of *dfoA,* the DFO synthetic gene, in E9 and these mutants. Both of these mutants produced reduced amounts of *dfoA* transcripts compared to E9 (Fig. 2). To further confirm that these phenotypes resulted from GacS and GacA deficiencies, we transferred the *gacA*$^+$ and *gacS*$^+$ cosmids into the cognate mutants. As was expected, EPS production (Fig. 1D) and swarming motility (1E) were restord in the mutants carrying the cognate genes (D2, D4, E2 and E4).

Effects of GacSA on expression of hrp genes

Previous studies have shown that *hrp* genes play critical roles in virulence of Ea. In light of global effects of GacS/GacA in many bacteria, it was of interest to examine the effects of GacSA deficiency on the expression of *hrp* and *dsp* genes. The data shown in Fig. 2 revealed that transcript levels of *hrpL* and *hrpN* genes are much reduced in the GacA$^-$ mutant AC5200 (column 2) and the GacS$^-$ mutant AC5201 (column 3) compared to the levels in the parent strain, E9 (column 1).

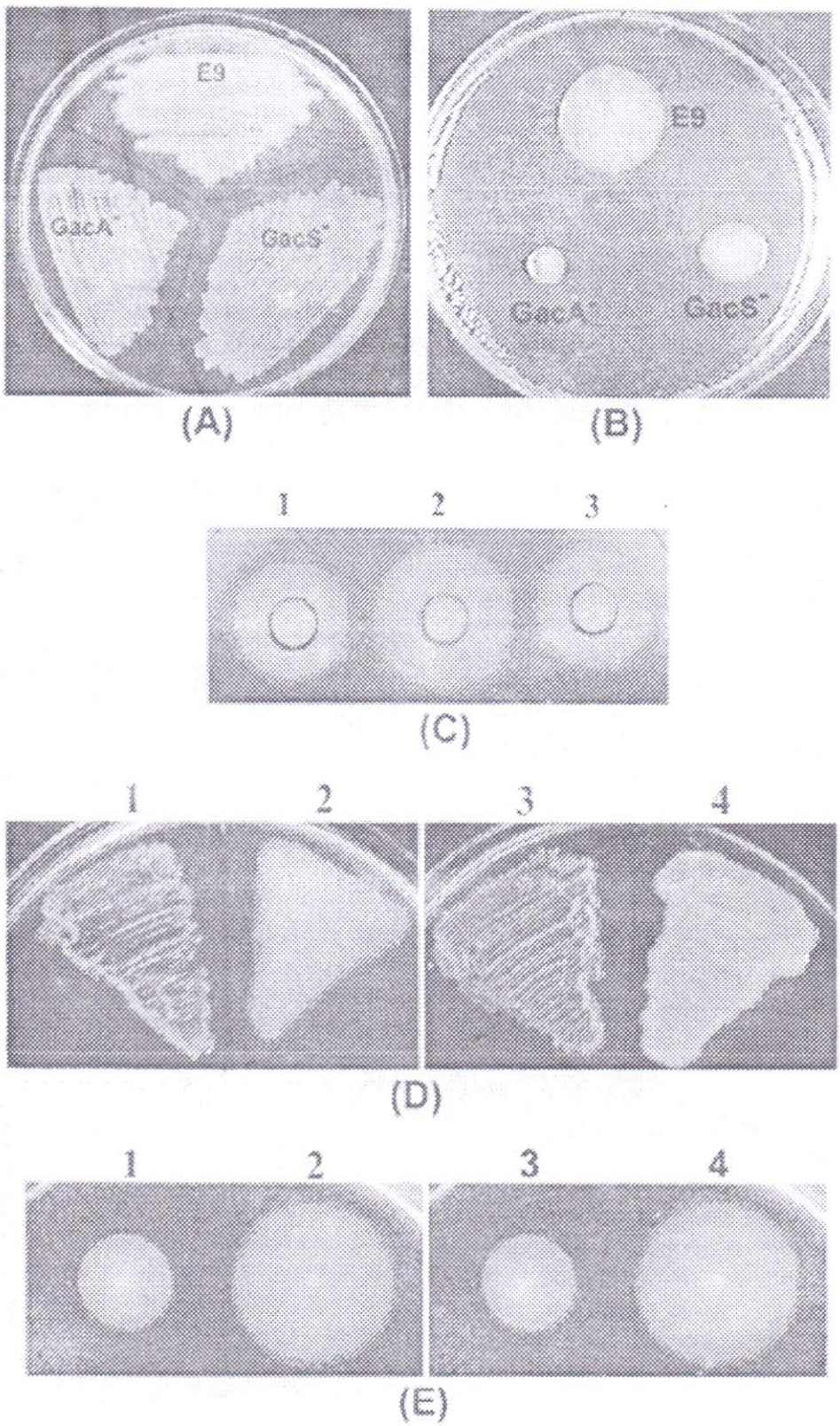

Fig. 1 : Characteristics of *E. amylovora* strain E9, its GacA⁻ and GacS⁻ mutants. (A), EPS production on KB agar, (B), swarming motility in soft KB agar (0.4%), and (C), siderophore production on CAS agar; (D) and (E), restoration of EPS production and swarming motility by gacA⁺ and gacS⁺ plasmids in E9 GacA⁻ and GacS⁻ mutants. 1, GacA⁻ mutant carrying the cloning vector pLARF5; 2, GacA⁻ mutant carrying the gacA⁺ plasmid; 3, GacS⁻ mutants carrying pLARF5 and 4, GacS⁻ mutants carrying the gacS⁺ plasmid.

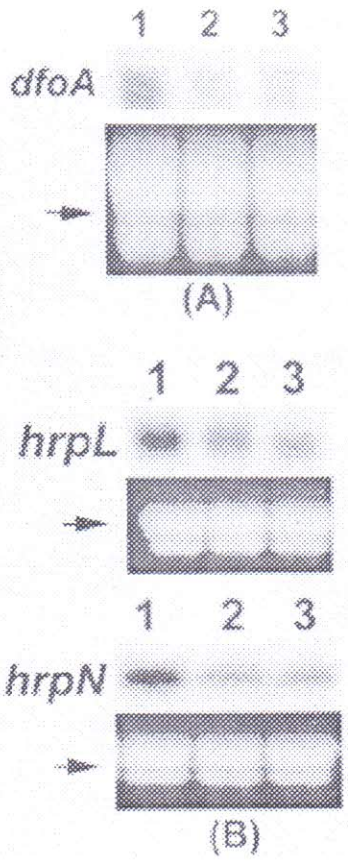

Fig. 2 : Transript levels of dfoA, hrpL and hrpN. 1, E9; 2, the GacA⁻ mutant and 3, the GacS⁻ mutant. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. Each lane contains 15 μg of bacterial total RNA.

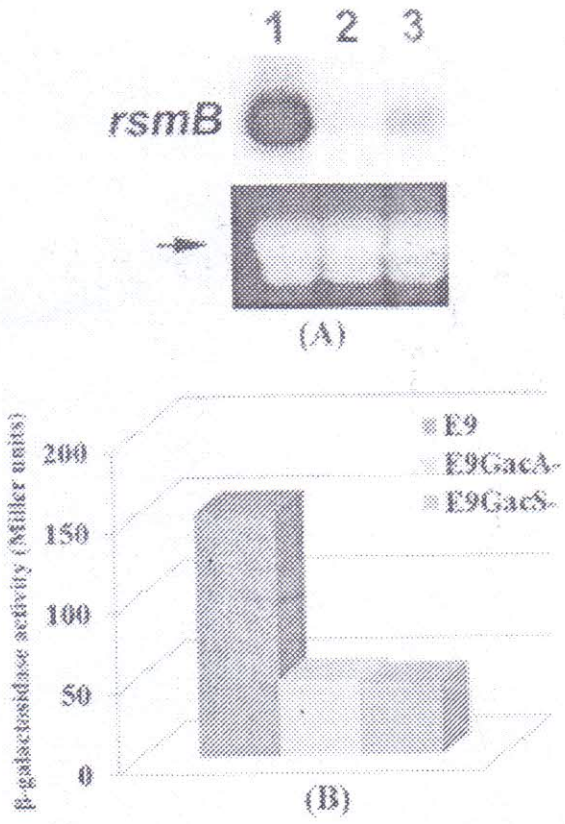

Fig. 3 : (A), rsmB RNA levels of 1, E9; 2, the GacA⁻ mutant and 3, the GacS⁻ mutant. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. Each lane contains 15 μg of bacterial total RNA, (B), β-galactosidase avtivity of E9, GacA⁻ mutant AC5200 and GacS⁻ mutant AC5201 carrying the transcriptional rsmB-lacZ fusion pAKC1047.

rsmB of E9:

CAGTATGTAATATGAGTGGGGAAGTCATCCTTGAGCAGTTGGGCACCAAAAAGCG
CCAGGTTATCGCGCCATCTATCCGGAACTTTCTTATTTCTTTACAGCCGCTTATC
ATTAATTATCCGAATTAAGCCGCCTGCCC**TGTACG**AGATCT**CTTACA**GATTATGT
AAGAGATCGCTTGTAATCATGCAGATATGACTAATGCTTAAGAATTAAAAATGAT
TCATATCATCTGGTTAGCCGATTTCAGGCCATCACAGAAGAAGGAATAGCCTAAA
TA

GCCGCTCTAAGCATCTTGTTTGCTAATGTTATTAAGGCACAATAGGCCCC**G**TTGC
GAAGGAACAGCATGATATGAGATTAACATCAGGATGATGTGCTCATTGAAGGATT
GAATCATCGGGATGATGTATCAGGGACAGGCTCCAGGATGGGGTACAGGAAACTT
CAGGAAGAGGTCAGGAACATCTCCAGGATGGAGAAACGCGCCGTAAGGGACATTC
GGCAGTCATGGATGATAACAGGATCACTATCAGGATGATATTTCAGGGACAGGCT
TCAGGATGAAGCAAAGGAGATCTCAGGAAGAGGTAATGGACACCTCCAGGACGGA
GAATGTGAGCCGTAAAGGACTATTGGCGGGCATGGAAGTCAAAGGATCGACGTCA
GGAAGATGTCTGCTCAGGAAAGCGCATGGACCAGTTTTCAAGGATGAGCAGGGAG
CATAAATGTAGCCGGATAGCTGCAAACGAACCGGGGGCACTGTTTATACAGTGCC
CCCTTTTTTT

Fig. 4 : Nucleotide sequence of E9 rsmB. The star indicates the transcriptional start site. The consensus GacA binding site is double underlined. The putative –10 and –35 regions are underlined.

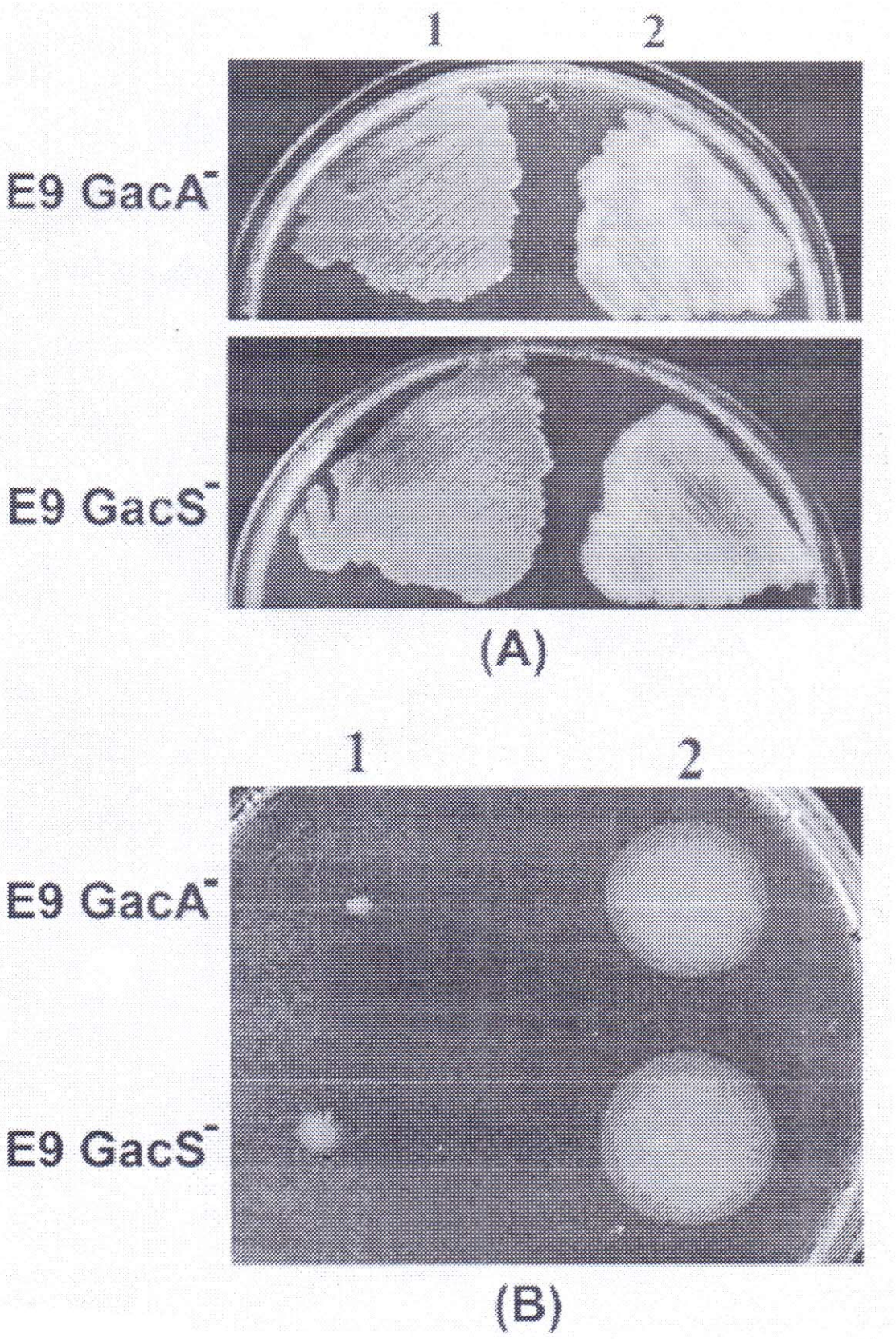

Fig. 5 : Restoration of (A) EPS production and (B) swarming motility by rsmB⁺ plasmid in E9 GacA⁻ and GacS⁻ mutants. 1, GacA⁻ or GacS⁻ mutant carrying the rsmB⁺ plasmid.

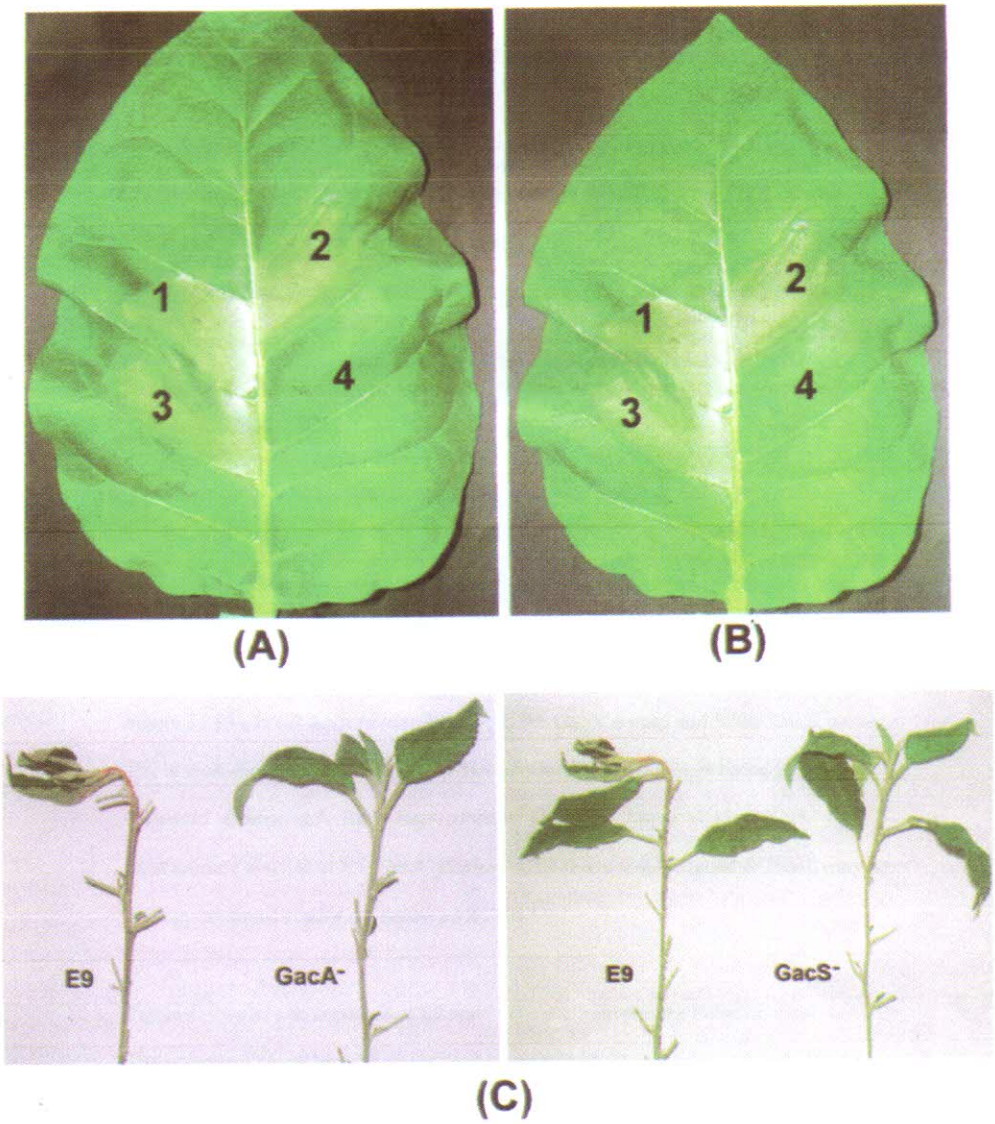

Fig. 6 : (A), HR symptoms elicited by E9 and its GacA⁻ mutant. (B) HR symptoms elicited by E9 and its GacS⁻ mutant. Site, E9 (2×10^8); site 2, GacA⁻ or GacS⁻ mutant (2×10^8); site 3, E9 (2×10^7) and site 4, GacA⁻ or GacS⁻ mutant (2×10^7). (C) disease symptoms produced in apple shoots by E9 and its GacA⁻ and GacS⁻ mutants.

Effects of GacS and GacA on rsmB RNA production

There is burgeoning evidence that the GacS/GacA two component system positively regulate *rsmB* or *rsmB*-like genes for sRNAs, and in fact recent studies have disclosed that this regulation is responsible for the phenotypes previously shown to be affected by GacSA. To assess the roles of GacSA in *rsmB* expression in Ea, we performed several experiments. First, we compared the levels of *rsmB* RNA in E9 and its GacA⁻ and GacS⁻ mutants. In the mutants *rsmB* RNA levels were barely detectable (Fig. 3A; lanes 2 and 3). This contrasts with high levels of *RsmB* transcript in the GacS/A⁺ parent (Fig. 3A; lane 1). To verify that this effect is due to inhibition of transcription, we introduced a previously made *rsmB-lacZ* transcriptional fusion (pAKC1047) into E9, the GacA⁻ mutant and the GacS⁻ mutant. We grew the constructs in KB medium and assayed for β-galactosidase activity. The levels of β-galactosidase in the mutants were much lower (31%) than in E9 (Fig. 3B). As stated above, the consensus sequence required for GacA-P (phosphorylated GacA) binding and activation of transcription has been postulated (Lapouge *et al.*, 2008). A search of the sequences upstream of putative –10 and –35 regions of Ea

rsmB revealed the presence of sequences with a near perfect match with the consensus sequence. This sequence in Ea *rsmB* is present 120 bases upstream of the transcriptional start site as shown in Fig. 4.

To confirm that the GacS/A effect was channeled via *rsmB,* we constructed an *rsmB*$^+$ plasmid in a low copy vector, pCL1920 (Lerner and Inouye, 1990). This construct was introduced into the GacA$^-$ and GacS$^-$ mutants and the plasmid carrying strains were tested for EPS production (Fig. 5A) and swarming motility (Fig: 5B). The phenotypes negatively affected by GacA and GacS deficiency were reversed by the presence of the *rsmB*$^+$ DNA (Fig. 5; columns A2 and B2). Thus, multiple copies *rsmB* DNA reversed the effects of GacA and GacS deficiency in Ea.

Effect of GacA and GacS on HR and pathogenicity

Since GacA and GacS affect virulence of Ecc and other bacteria and in light of their effects on the expression of *hrp* and *dsp* genes, it was of interest to determine the roles of *gacA* and *gacS* in the HR and pathogenicity of Ea. There was significant difference between the wild type strain and GacA$^-$ or GacS$^-$ mutant in terms of induction of the HR on tobacco and pathogenicity on apple shoots. Wild type strain E9 induced the HR when 2×10^7 cfu/ml was introduced into tobacco leaves whereas the GacA$^-$ mutant (Fig. 6A, site 4) or the GacS$^-$ mutant (Fig. 6B site 4) failed to induce the HR at this cell density. However, when 2×10^8 cfu/ml was introduced, both mutants induced HR like the parent strain, E9 (Fig. 6A sites 1, 2, 3 and Fig. 6B 1,2, 3).

GacA$^-$ and GacS$^-$ mutants required ten times higher cell density to induce the fire blight on apple shoots (Fig. 6C). Thus, deficiency of GacSA impairs virulence but does not abolish it.

DISCUSSION

In this report we have for the first time demonstrated that GacS/A function as global regulators in Ea controlling EPS and siderophore production, motility and determinants of pathogenicity and HR. We also present evidence supporting the hypothesis that this regulation is mediated via post-transcriptional regulators: a regulatory RNA (*rsmB*) and an RNA binding protein (RsmA) [see below for the evidence for the latter].

GacS/A mediated regulation has been extensively studied in *Erwinia* and *Pseudomonas* species (see Lapouge *et al.,* 2008 and references cited therein). Consequently, we can predict the details of molecular events including the targets of GacA-mediated activation of transcription. In *E. carotovora* ssp *carotovora, Pseudomonas syringae* pathovars, *P. fluorecens* and *P. aeruginosa,* GacA activates transcription of sRNA genes (Chatterjee *et al.,* 2003; Cui *et al.,* 2001; Lapouge *et al.,* 2008). In this work we have extended those observations to the fire-blight pathogen, *E. amylovora.* Several lines of evidence demonstrate that GacA activates rsmB RNA production and this effect in fact is responsible for the pleiotropic phenotype resulting from GacS or GacA deficiency. The evidence includes: remarkable reduction in *rsmB* RNA production in the mutants; inhibition of expression of a transcriptional *rsmB-lacZ* fusion; and the reversal of effects of GacS/A deficiency by increasing the dosage of *rsmB* DNA. Moreover, the promoter region of *rsmB* of Ea contains the putative GacA binding sequences in close proximity to the –10 and –35 regions. The near perfect match with the consensus sequence and the location of the putative binding sequences strongly suggest molecular interaction between GacA (GacA-P) with this DNA region. By drawing upon the findings with other systems and based upon the data presented here, we propose a model that depicts a GacS/A regulatory pathway in the fire-blight pathogen.

Studies with *E. amylovara* (Ma *et al.,* 2001), *E. coli* (Liu *et al.,* 1997). *E. carotovora* ssp *carotovora* (Liu *et al.,* 1998). and *Pseudomonas* species (Lapouge *et al.,* 2008) have revealed that *rsmB* RNA acts as a positive regulator by binding RsmA or RsmA-like RNA binding proteins responsible for promoting RNA decay. The observation that overexpression of *rsmB* reverses the effects of GacA and GacS deficiency, and since the primary if not the sole effect of *rsmB* is to counteract the effects of RsmA, we predicted that in GacS$^-$ and GacA$^-$ mutants, the levels of *rsmB* RNA would be low and not enough to fully quench the free RsmA molecules. We suggest that the low levels of transcripts of *hrpL* and other genes in the GacA$^-$ mutant (Fig. 2) are due to a more rapid decay of the transcripts than in the GacA$^+$ strain. These observations collectively support the hypothesis that this two component

system in *E. amylovora* works via post-transcriptional regulators.

In general, GacS/A affect traits required for plant interaction, ecological fitness and secondary metabolite production, without serious ramifications in the expression of house keeping genes. This preferential effect could be attributed to the characteristics of RsmA action. As stated above, the net result of GacS/A retulation is *rsmB* RNA production thereby modulating the cellular concentration of free RsmA. It has also become apparent that transcripts of genes for secondary metabolites, pathogenicity determinants as well as those expressed in a growth phase/cell density dependent manner, as opposed to transcripts of housekeeping genes, are more susceptible to RsmA action. The basis for this preferential interaction is not known. It is possible that RNA structure may influence RsmA-RNA interaction and the subsequent fate of such complexes.

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