GacA, the Response Regulator of a Two-Component System, Acts as a Master Regulator in *Pseudomonas syringae* pv. tomato DC3000 by Controlling Regulatory RNA, Transcriptional Activators, and Alternate Sigma Factors

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GacA, the Response Regulator of a Two-Component System, Acts as a Master Regulator in Pseudomonas syringae pv. tomato DC3000 by Controlling Regulatory RNA, Transcriptional Activators, and Alternate Sigma Factors

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Concerted investigations of factors affecting host-pathogen interactions are now possible with the model plant Arabidopsis thaliana and its model pathogen Pseudomonas syringae pv. tomato DC3000, as their whole genome sequences have become available. As a prelude to analysis of the regulatory genes and their targets, we have focused on GacA, the response regulator of a two-component system. The DC3000 gene was cloned by testing for the reversal of phenotypes of an Erwinia GacA− mutant. A GacA− mutant of DC3000 constructed by marker exchange produces much-reduced levels of transcripts of three alternate sigma factors: HrpL, required for the production of effector proteins and their translocation via the type III secretion system; RpoS, required for stress responses and secondary metabolism production; and RpoN, required for an assortment of metabolic processes and expression of hrlP. GacA deficiency also reduces the expression of hrpR and hrpS, which specify enhancer-binding proteins of the NtrC family required for hrpL transcription; ahlI and ahlR, the genes for quorum sensing signal; salA, a regulatory gene known to control virulence; CorS, a sensor kinase; CorR, the cognate response regulator that controls coronatine biosynthetic genes; and rsmB and rsmZ, which specify untranslatable regulatory RNA species. gacA expression itself is regulated by environmental conditions in DC3000, since transcript levels are affected by growth phase and media composition. The observations that high levels of gacA RNA occur in the hrp-inducing medium and GacA deficiency reduces the level of rpoS expression implicate an important role of GacA in stress responses of DC3000. Consistent with the effects on hrpL expression, the GacA− mutant produces lower levels of transcripts of avr, hrp, and hop genes controlled by HrpL. In addition, GacA deficiency results in reduced levels of transcripts of several HrpL-dependent genes. As would be expected, these effects on gene expression cause drastic changes in bacterial behavior: virulence towards A. thaliana and tomato; multiplication in planta; efficiency of the induction of the hypersensitive reaction (HR); production of pigment and N-acyl-homoserine lacco}

Of the plethora of regulators identified in pathogenic and environmentally significant bacteria, members of the two-component system are perfectly suited to sense and recognize environmental cues and signals to transduce the information to transcriptional factors, which could, in turn, activate the expression of one or several genes whose production allows the bacterium to rapidly adjust to environmental conditions. Such systems generally comprise two proteins: a sensor kinase that perceives a signal or cue, autophosphorylates, and then activates its partner, a protein known as a response regulator (RR). The activated RR functions as the transcriptional activator of one or more genes. A two-component system comprising GacS and GacA or their homologs has been found in numerous bacteria, including plant pathogens (Heeb and Haas 2001). In this case, GacS is the putative sensor kinase and GacA is the RR. This system has been found to control an array of phenotypes, including pathogenicity, plant growth promoting capability, survival, production of secondary metabolites such as antibiotics, quorum sensing signals and toxins, motility, biofilm formation, secretion systems, extracellular polysaccharides, and secreted proteins and enzymes (Blumer et al. 1999; Bull et al. 2001; Castañeda et al. 2001; Chancey et al. 1999 2002; Cui et al. 2001; Duffy and Défago 2000; Heeb and Haas 2001; Laville et al. 1992; Natsch et al. 1994; Parkins et al. 2001; Pessi and Haas 2001; Rich et al. 1994; Saleh and Glick 2001; Whistler et al. 2000; Willis et al. 1990).

Despite extensive studies of phenotypic effects of GacS/GacA in various bacteria, little is known about the modes of action of the two components, the nature of the signals perceived by GacS, regulation of GacA production and modulation of GacA function, the primary targets of GacA, and the inventory of the genes and their products that are controlled by GacA. To alleviate these deficiencies, we have chosen to study the GacS/GacA system of the model pathogen Pseudomonas syringae pv. tomato DC3000, which infects tomato as well as Arabidopsis thaliana. In this report, we present evidence that
GacA is a master regulator that controls several alternate sigma factors, genes for quorum sensing signal and regulatory RNA and protein species. Our finding that GacA controls HrpL-dependent genes required for virulence as well as regulators of the chlorosis-inducing toxin coronatine is consistent with its role as a global regulator of virulence determinants. Moreover, the effects of GacA on quorum-sensing signal and alternate sigma factors have important ramifications in ecological fitness of this plant pathogen. We also document for the first time that GacA production is regulated in DC3000 by growth phase and stress conditions.

Studies in Erwinia carotovora (Chatterjee et al. 1995; Cui et al. 1995), Escherichia coli (Romeo 1998), Pseudomonas aeruginosa (Pessi et al. 2001), and P. fluorescens (Blumer et al. 2000; Heeb et al. 2002) have revealed that posttranscriptional regulation mediated by the RsmA–RsmB pair (CsrA and csrB in E. coli) plays a critical role in gene expression. RsmA is a small RNA-binding protein that acts by repressing translation and by promoting decay of mRNA species. rsmB specifies an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect. It also has become apparent that GacS and GacA control rsmB expression, and genetic data demonstrate that this regulation is, in fact, responsible for the GacA effect on various phenotypes in Erwinia carotovora (Cui et al. 2001). We have determined the presence of rsmA, rsmB, and rsmZ genes in DC3000, and our findings with cloned genes have revealed that several RsmA species function as negative regulators and that rsmB RNA and rsmZ RNA act as positive regulators (A. Chatterjee, unpublished data). We document here that GacA positively controls rsmB and rsmZ RNA production in DC3000. Based on the findings reported here, we present a model depicting the GacA regulon of DC3000.

RESULTS

Sequence analysis of gacA DC3000

Analysis of the whole genome sequence of P. syringae pv. tomato DC3000 revealed the presence of a homolog of gacA comprising a 666-bp open reading frame that could encode a putative GacADC3000 polypeptide of 24.2 kDa with 222 amino acid residues. A Blast search revealed that GacADC3000 shares high homology with GacA of P. syringae pv. syringae (92% identity and 93% similarity) (accession number A55538), GacA of P. fluorescens (85% identity and 89% similarity) (accession number BAB41136.1), GacA of P. aeruginosa (84% identity and 89% similarity) (accession number NP_251276.1), and GacA of P. viridiflava (91% identity and 93% similarity) (accession number AAB38979.1). The results of a Prosise scan of GacADC3000 revealed a putative response regulatory domain located between amino acid residues 11 and 127, a putative HTH motif of the LuxR family located between residues 172 and 199, and seven putative protein kinase C phosphorylation sites. Domains and motif are conserved in GacA species of several other bacteria (Heeb and Haas 2001). Northern blot analysis using an internal fragment of gacADC3000 (polymerase chain reaction [PCR] product) as a probe revealed an approximately 0.75-kb transcript in DC3000 (Fig. 1A, lane 1), indicating that gacADC3000 comprises a single gene operon. As would be expected, the GacADC3000 mutant AC811, constructed by marker exchange, does not produce this RNA species (Fig. 1A, lane 2). Sequence analysis of pAKC1112 (inactive gacA plasmid) (Table 1) revealed that the mini-Tn5-Km insertion is located at 361 nt from the translational start site. The transcriptional start site of gacADC3000 was located by S1 nuclease protection analysis to the C residue at base –79 relative to the translational start site (data not shown).

Media and cell density effects on expression of gacA

To examine the effects of media and growth rate on the expression of gacADC3000, total RNA samples were extracted from DC3000 grown in rich medium (King’s B [KB]) at Klett values of 100, 200, and 300 or grown in KB medium to a Klett value of 100 and transferred into hpr-inducing medium (IM) and incubated for an additional 0.5, 1, 1.5, and 2 h. Northern blot analysis with gacADC3000 probe (Fig. 1B) revealed that: i) the levels of gacADC3000 transcript were higher at higher cell densities in KB medium and ii) the gacADC3000 transcript levels were higher when incubated in IM than in KB medium, and it reached the highest level after 2 h incubation in IM.

GacA positively controls the production of AHL.

Search of the DC3000 genome sequence database revealed the existence of homologs of ahlR and ahlI genes that encode members of the LuxR-LuxI family of regulatory proteins. By using an E. coli-based bioassay system (Chatterjee et al. 1995), we detected N-acyl-homoserine lactone (AHL) in spent cultures of DC3000 and its GacADC3000 mutant. DC3000 produced 1.3 × 10^7 relative light units (RLU), and its mutant,
AC811, produced 1.0 × 10^6 RLU when grown in KB medium for 20 h. Northern blot analysis of total RNAs from cultures incubated in IM medium also revealed that the transcript levels of ahlII and ahlR were much lower in AC811 than in DC3000 (Fig. 1C).

Effects of GacADC3000 on growth, motility, and pigment and siderophore production.

To test the effect of GacA DC3000 on growth, motility, and pigment and siderophore production, the GacADC3000 mutant AC811 and its parent were grown in minimal salts plus succinic acid (disodium salt, 0.5% wt/vol) medium, KB medium, SWM agar plate (Kinscherf and Willis 1999), and chrome azurolS (CAS) agar plate (Schwyn and Neilands 1987). The growth curves (Fig. 2) of DC3000 and AC811 in minimal salts plus succinic acid and KB revealed that: i) the mutant reached stationary phase at a slightly lower cell density compared with its parent in both KB and minimal media and ii) the doubling time (e.g., time required for growth from Klett values of 50 to 100) of DC3000 was approximately 2 h in both of the media, whereas the mutant has a doubling time of approximately 2 h in KB and 2.5 h in minimal salts plus succinic acid.

DC3000, like many other Pseudomonas species, produces diffusible yellow pigment on KB agar medium. As shown in Figure 3A, the GacA– mutant AC811 is much lighter in color than its parent.

To examine the GacADC3000 effect on motility, DC3000 and its GacADC3000– mutant were patched on SWM medium and were incubated for 12 h at 28°C. The results shown in Figure 3B reveal that the swarming motility of the mutant is much reduced compared with that of DC3000.

To test the effect of GacA on the siderophore production, DC3000 and its GacADC3000– mutant AC811 were grown on CAS agar plate. Contrary to pigment production and swarming motility, GacADC3000 deficiency does not affect siderophore production (data not shown).

GacADC3000 affects the capacity to elicit the hypersensitive response (HR).

The results in Figure 3C show that DC3000 elicited typical HR in tobacco leaf (Nicotiana tabacum L. cv. Samsun) when infiltrated with 1 × 10^6 CFU per ml of bacterial cells (Fig. 3C, site 1), whereas the GacADC3000 mutant failed to elicit the HR symptom at that cell concentration (Fig. 3C, site 2). Both the mutant and parent induced the HR when 1 × 10^8 CFU per ml of bacterial cells were infiltrated (data not shown).

Effects of GacADC3000 on pathogenicity.

To compare the effects of GacADC3000 on pathogenicity, we tested the responses of DC3000 and its GacADC3000 mutant in Arabidopsis and tomato plants. Arabidopsis leaves were infiltrated with 1 × 10^6 CFU per ml of bacterial cells of DC3000 or its GacA– mutant, AC811. Tomato leaves were dip-inoculated in 2 × 10^7 CFU per ml of bacterial cell suspensions. Figure 3D shows that DC3000 produced disease symptoms in Arabidopsis leaves. In contrast, the GacADC3000– mutant failed to produce any disease. The results shown in Figure 3E reveal that the severity of symptom production in tomato leaves by the GacADC3000– mutant (right) was much reduced as compared with DC3000 (left). This observation correlated with bacterial cell population in tomato leaves (Fig. 3F) in that the population of the mutant cells was lower than that of the parent for two to five days postinoculation.

GacADC3000 controls regulatory genes.

Alternate sigma factors. HrpL, an alternate sigma factor, plays a central role in the production of the type III secretion system and the proteins (= effectors) secreted by this pathway (Chatterjee et al. 2002; Collmer et al. 2000, 2002; Fouts et al. 2002; Frederick et al. 2001; Mor et al. 2001; Petnicki-Ocwieja et al. 2002; Wei and Beer 1995; Wei et al. 2000; Xiao et al. 1994). The results shown in Figure 4A reveal that the transcript levels of hrpL and rpoN (sigma54), an alternate sigma factor required for expression of hrpL (Chatterjee et al. 2002;

Table 1. Bacterial strains and plasmids used in this research

<table>
<thead>
<tr>
<th>Strain / plasmid</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td>Erwinia carotovora subsp. carotovora Ecc71</td>
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<td>Zink et al. 1984</td>
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<td>AC5056</td>
<td>Km, GacA– derivative of Ecc71</td>
<td>Cui et al. 2001</td>
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<tr>
<td>AC5088</td>
<td>Cm, RsmB derivative of Ecc71</td>
<td>Chatterjee et al. 2002</td>
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<td><em>Pseudomonas syringae</em> pv. tomato DC3000</td>
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<td>A. Collmer</td>
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<td>AC811</td>
<td>Km, GacADC3000– derivative of DC3000</td>
<td>This work</td>
</tr>
<tr>
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<td>Wild type</td>
<td>K. Willis</td>
</tr>
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<td>BGACX</td>
<td>Km, GacA– derivative of B728a</td>
<td>Rich et al. 1994</td>
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<td>Chatterjee et al. 1996</td>
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<td>Tc, vector</td>
<td>Windgassen et al. 2000</td>
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<td>pKnockoutΩ</td>
<td>Sp, vector</td>
<td>Fürste et al. 1986</td>
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<tr>
<td>pMMB66EH</td>
<td>Ap, vector</td>
<td>This work</td>
</tr>
<tr>
<td>pMMB66EHΩ</td>
<td>Sp, Sp cassette inserted at PvuI site of pMMB66EH</td>
<td>This work</td>
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<td>de Lorenzo et al. 1990</td>
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<td>pAKC1111</td>
<td>Tc, GacADC3000– from DC3000 genomic library in pCPP47</td>
<td>This work</td>
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<td>pAKC1112</td>
<td>Km, Tc, GacADC3000–, pAKC1111 mutagenized by mini-Tn5-Km</td>
<td>This work</td>
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<td>J. Alfano</td>
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<td>pLN8</td>
<td>Sp, 700 bp of hopPtoL in pKnockoutΩ</td>
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<td>pLN9</td>
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<td>Ap, off in pBluescript SK+</td>
<td>C. Bender</td>
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Hendrickson et al. 2000), are much reduced in the GacADC3000– mutant compared with those in its parent.

Contrary to the results of DC3000, the elicitation of the HR is not affected by GacA in P. syringae pv. syringae B728a (Willis et al. 1990). Northern blot analysis results (Fig. 4B) revealed that the transcript levels of hrlP are very similar in B728a and its GacA– mutant BGACX.

Stationary-phase sigma factor \( \sigma^c \) (rpoS) is positively regulated by GacA in P. fluorescens and E. coli (Mukhopadhyay et al. 2000; Whistler et al. 1998). Our data (Fig. 4A) show a similar effect of GacADC3000 on the expression of rpoS in DC3000. However, the expression of algT (encoding sigma E) is not affected by GacADC3000 (Fig. 4A).

**hrpRS, regulatory genes controlling hrpL expression.** In P. syringae pv. tomato as well as P. syringae pv. syringae, hrpL is controlled by the NtrC-like regulators HrpRS that, in conjunction with sigma-54, activate the hrpL promoter (Hendrickson et al. 2000). In light of the effects of GacA on hrpL RNA levels (discussed above), the effect of GacA deficiency on the expression of hrpRS genes was tested. The results of Northern blot analysis (Fig. 4C) showed that both hrpR-specific and hrpS-specific probes hybridized to the same 2.1-kb RNA species of DC3000. The 1.1-kb RNA species, like the 2.1-kb RNA, carries sequences for both the genes and is presumed to represent a relatively stable degradation product. These data confirm the observations of Hutcheson and associates (2001) that hrpR and hrpS compose an operon. With the hrpR-specific probe, we noted hybridization with an approximately 0.5-kb RNA species (Fig. 4C, band c) that most likely is the processed product of the hrpRS transcript. We do not yet know if hrpR and hrpS are differentially expressed or if RNA processing plays a role in such expression. This uncertainty notwithstanding, our data clearly establish that the GacA– mutant, compared with the GacA+ parent, makes much-reduced levels of the 2.1-kb hrpRS transcript and the RNA species derived from it.

**SalA, a DNA-binding regulatory protein.** In P. syringae pv. syringae, the production of syringomycin as well as virulence are controlled by SalA (Kitten et al. 1998; Lu et al. 2002). Our results revealed that, in DC3000 and AC811, the transcript levels of salA were higher in IM medium than in KB medium (Fig. 4D, lanes 1 and 3) and that compared with DC3000, the transcript levels of salA in GacADC3000– mutant were reduced in both KB and IM media (Fig. 4D, lanes 2 and 4 compared with lanes 1 and 3, respectively).

**corR, corS, and coronatine biosynthetic genes.** Since coronatine is an important virulence factor (Bender et al. 1999; Ullrich et al. 1995) and GacA affects virulence of DC3000 (Fig. 3D and E), the effect of GacA on coronatine regulatory and biosynthetic gene expression was examined. Extensive studies by Bender and associates (Bender et al. 1999; Peña-loza-vázquez and Bender 1998; Rangaswamy and Bender 2000; Wang et al. 1999) and that of Smirnova et al. (2002) have established that CorS and CorR, members of a modified two-component system, control the biosynthetic genes. A Blast search of the DC3000 whole genome sequence disclosed the presence of corS and corR sequences. CorS is predicted to function as a sensor kinase, whereas CorR is believed to function as a response regulator. To assess the roles of GacA on these putative cor regulatory genes, we conducted Northern blot assays (Fig. 4E). It is evident that corR and corS RNA levels are reduced more in the GacA– mutant than in DC3000. Consistent with this observation was the effect of GacA deficiency on several biosynthetic genes (Fig. 5). The GacA– mutant produced barely detectable levels of transcripts of cfl, a gene for coronafacate ligase, as well as of cfa8 and cfa9, genes involved in coronatine biosynthesis. These data clearly contrast with high levels of transcripts of these cor genes in DC3000.

**rsmB and rsmZ RNA species.** rsmB, a nontranslatable RNA molecule, belongs to the RsmA–rsmB posttranscriptional regulatory system. It binds RsmA, an RNA binding protein promoting mRNA decay, and neutralizes the action of RsmA. Our previous work in Erwinia carotovora revealed that GacA positively regulates rsmB.

In P. fluorescens, rsmZ (prrb), which is structurally similar to rsmB, was found to be positively controlled by GacA (Aarons et al. 2000; Heeb et al. 2002). Overexpression of rsmZ increased the production of Phl (2, 4-diacyethylphloroglucinol) and hydrogen cyanide (Aarons et al. 2000; Heeb et al. 2002).

To assess the regulatory effects of GacA on rsmB and rsmZ in DC3000, we cloned a rsmBDC3000 and a rsmZDC3000 locus from a DC3000 genomic library. The Northern blot data (Fig. 4F) show that DC3000 produced high levels of rsmB and rsmZ transcripts, whereas the mutant produced barely detectable levels of rsmB and reduced levels of rsmZ transcripts.

**Effects of GacADC3000 on the transcript levels of hrpL-dependent and hrpL-independent genes.**

Our data demonstrated that the transcript levels of hrpL are positively regulated by GacADC3000 (Fig. 4A), most likely due to its effects on hrpRS and rpoS. Recent studies have established a fundamental role of HrpL in the expression of many hrp, hrc, and hop genes and virulence effector genes known or predicted to possess the HrpL promoter (Boch et al. 2002; Chatterjee et al.

![Fig. 2. Growth curve of DC3000 and its GacADC3000– mutant AC811 in King’s B medium and minimal salts plus succinic acid (0.5% wt/vol) media.](image-url)
To test the effect of GacA deficiency, we determined the transcript levels of HrpL-dependent genes in DC3000 and its GacA^DC3000^ mutant (Fig. 5A). It is apparent that RNA levels of the following genes were much lower in the mutant: i) hrpZ, encoding harpin and hrpC, which specifies a component of the type III secretion system; ii) eight virulence effector genes (hopPtoD1, hopPtoD2, hopPtoC, avrPpiB, hopPtoF, hopPtoI, hopPtoK, and avrPtoB); iii) hopPtoM and hopPtoN, which are located within the pathogenicity island; and iv) hopPmaH, encoding a pectin lyase. By contrast, in P. syringae pv. syringae B728a, the transcript levels of hrpZ are not affected by GacA deficiency (Fig 4B).

Recent observations have revealed that several genes possessing HrpL-independent promoters are required for pathogenicity and virulence of DC3000 (Boch et al. 2002). This finding, taken along with a severe negative effect of GacA deficiency on pathogenicity, prompted us to test the effects of
GacA on several hrpL-independent genes. RNA levels of fliO, a gene encoding flagellar protein, and cel orfI, a gene encoding lytic murein transglycosylase, were also lower in the mutant compared with that of DC3000. Likewise, topA, the gene encoding transposase, and fadD, the gene for long-chain fatty acid CoA ligase, presumed not to affect pathogenicity, were also negatively affected by GacA deficiency. By contrast, the transcript levels of catF, a gene encoding catalase, and algD, a gene encoding GDP mannose dehydrogenase are not affected by GacA (Fig. 5B).

**Restoration of GacA** Controlled traits in AC811 by gacADC3000 plasmid.

To rigorously test that the phenotypic changes in AC811 resulted from inactivation of gacADC3000, we constructed pAKC1114, which contains the gacADC3000 DNA as well as 372 bp upstream in pRK415. This plasmid or pAKC1111 containing DC3000 gacA and flanking DNA in pCPP47 were transferred into AC811 and tested for various phenotypes. The results revealed that pigment production (Fig. 3G), motility (Fig. 3H), and capacity to elicit the HR (Fig. 3I and J) were restored to the wild-type level by pAKC1111 or pAKC1114 in AC811. The transcript levels of salA, rsmB, rsmZ, hrpL, hrpZ, and hopPtoC were also higher in AC811 carrying pAKC1111 or pAKC1114 than in AC811 carrying the cloning vectors (Fig. 4G). Since pAKC1114 carries only the gacADC3000 DNA, we conclude that the phenotypes changed in AC811 due to GacA deficiency.

**DISCUSSION**

In this report, we have presented significant new findings on the effects of GacA, a response regulator of a two-component system of *P. syringae* pv. tomato DC3000. Our data reveal that GacA plays a central regulatory role by controlling the production of various alternate sigma factors, quorum sensing signal and regulatory RNA, and protein species. The regulatory scheme depicted in Figure 6 places GacA at the top in the regulatory hierarchy, based upon the effect of GacA on regulators known or predicted to control expression of various bacterial traits. The observations that are especially relevant to the biology and ecology of this bacterium include effects on movement, production of secondary metabolites, including the putative quorum sensing signal, overall performance as a pathogen, and capacity to produce virulence effectors and to elicit host defense responses. We discuss below the findings in light of

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**Fig. 4.** A, Transcript levels of hrpL, rpoN, rpoS, and algT of DC3000 and AC811. Column 1, DC3000 and column 2, AC811. B, Transcript levels of hrpL and hrpZ of *P. syringae* pv. syringae B728a (column 1) and its GacA- mutant DBGCX (column 2). C, Transcript levels of hrpR and hrpS of DC3000 and AC811. Column 1 and 3, DC3000 and column 2 and 4, AC811. The arrow head shows the level of total RNA as revealed by ethidium bromide staining of the denaturing agarose gel. D, Transcript levels of salA of DC3000 and AC811 in King’s B (KB) medium (lanes 1 and 2) and hrp-inducing medium (IM) (lanes 3 and 4). Lanes 1 and 3, DC3000 and lanes 2 and 4, AC811. E, Transcript levels of corS and corR of DC3000 (column 1) and AC811 (column 2). F, Effects of GacA on the transcript levels of rsmB and rsmZ. Column 1, DC3000 and column 2, AC811. G, Transcript levels of salA, ahfI, rsmB, rsmZ, hrpL, hrpZ, and hopPtoC in AC811 carrying the vector pCPP47 (column 1), the GacA plasmid pAKC1111 (column 2), the vector pRK415 (column 3) and the GacA plasmid pAKC1114 (column 4). For rpoN, salA (lanes 1 and 2 of D), total RNAs were obtained from bacteria grown in KB medium to a Klett value of approximately 100 and incubated in hrp-inducing medium for an additional 2 h. Each lane contained 15 µg of total RNA.

**Fig. 5.** Effects of GacA on the transcript levels of A, HrpL-dependent genes and B, HrpL-independent genes. Total RNAs were obtained from bacteria grown in King’s B medium to a Klett value of approximately 100 and incubated in hrp-inducing medium for an additional 2 h. Each lane contained 15 µg of total RNA. Column 1, DC3000 and column 2, AC811.
GacADC3000 shares high homology with GacA proteins of various Pseudomonas species and, like the other GacA species, it contains a putative response regulatory domain within the N-terminal, a putative HTH motif of the LuxR family, likely involved in DNA binding in the C-terminal. The C-terminal HTH motif is predicted to determine DNA recognition specificity of GacA. Mutational analyses of gacADC3000, especially mutations in these domains, are needed to define their functions in regulation of gene expression. With regard to the primary target of GacADC3000, our findings, albeit somewhat preliminary, make a case for rsmB and rsmZ RNA. First, the expression of rsmB and rsmZ is severely reduced in the absence of GacA. Second, multiple copies of rsmB reverse the effects of GacA deficiency; this reversal is more pronounced when a non-GacA-responsive promoter replaces the native promoter (A. Chatterjee, unpublished data). We should note that rsmB is a putative HTH motif of the LuxR family, likely involved in DNA binding in the C-terminal. The C-terminal HTH motif is predicted to determine DNA recognition specificity of GacA. Mutational analyses of gacADC3000, especially mutations in these domains, are needed to define their functions in regulation of gene expression. With regard to the primary target of GacADC3000, our findings, albeit somewhat preliminary, make a case for rsmB and rsmZ RNA. First, the expression of rsmB and rsmZ is severely reduced in the absence of GacA. Second, multiple copies of rsmB reverse the effects of GacA deficiency; this reversal is more pronounced when a non-GacA-responsive promoter replaces the native GacA-responsive rsmB promoter (A. Chatterjee, unpublished data).

While GacA affects a large array of traits (Heeb and Haas 2001), the specific traits affected in different bacterial species or subspecies may differ. In P. aeruginosa PA14 (Parkins et al. 2001), GacA was found to affect biofilm formation but not flagellar-mediated attachment, solid surface translocation by pili, autoinducer production, or alginate biosynthesis. This contrast with the effects of GacA on swarming motility and AHL production by DC3000 as reported here. GacA and GacS were found to control quorum sensing signal in P. aureofaciens (Chancey et al. 1999) and P. syringae pv. syringae (Dumenyo et al. 1998; Kinscherf and Willis 1999; Kitten et al. 1998) as well. Alginate production is controlled by GacS and GacA in Azotobacter vinelandii (Castañeda et al. 2001) and P. syringae pv. syringae B728a (Willis et al. 2001). However, in DC3000, no effect of GacA on algT and algD transcript levels were seen (Figs. 4A and 5B). In P. syringae pv. syringae B728a (Willis et al. 1990), GacA and GacS affect pathogenicity but not HR. This finding contrasts with the effects of GacA on both HR and pathogenicity of DC3000 (discussed below). Our observations suggest that such differences reflect the composition of the GacS and GacA regulatory cascade, i.e., the nature of the downstream regulatory genes that are affected by GacS and GacA.

In the context of plant interaction of DC3000, the regulation of hrpL, salA, corR, and corS by GacA is especially critical. The pioneering work of N. Panopoulos (Rahme et al. 1991) and subsequent elegant studies of S. W. Hutcheson (Bretz et al. 2002; Hutcheson et al. 2001) established the regulatory cascade involved in the expression of hrpL. hrpL transcription is activated by HrpR and HrpS, regulators belonging to the NtrC family of enhancer-binding proteins, in conjunction with σ^44. Here, we have documented that GacA controls the expression of hrpRS and rpoN, and these findings strongly suggest that the regulatory effect of GacA on hrpL is a consequence of these regulations. In addition to confirming the operon organization of hrpRS in DC3000 (Hutcheson et al. 2001), our data raise the possibility of processing of the transcripts yielding predominantly RNA carrying hrpR sequences. We should note that RNA processing as a means of differential gene expression has been reported for various bacterial operons.

Studies with several Erwinia and Pseudomonas species, including DC3000, have disclosed that HrpL plays a critical role in bacteria-plant interactions. It positively controls the expres-

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**Fig. 6.** Schematic depiction of the GacS/A regulon of Pseudomonas syringae pv. tomato DC3000. GacS, the putative sensor kinase, is presumed to respond to cues generated during the postexponential bacterial growth phase and under various physiological and stress conditions. It is likely that, under these conditions, GacS autophosphorylates and then activates GacA. The activated GacA positively regulates the production of several alternate sigma factors, DNA binding proteins, and regulatory RNA species. These observations establish a central regulatory role of GacS and GacA, and place these higher in the regulatory hierarchy. The effects of GacA on various regulators are known or predicted to cause profound changes in bacterial behavior, including movement, pathogenicity, elicitation of defense responses, stress responses, and the production of toxins, pigments, and the putative quorum sensing signal. Several major components, RpoN, HrpL, SalA, and CorS and CorR, known to affect genes for plant interaction, i.e., vir, avr, hrp/hop, as well as cor and cfa, are all regulated by the GacS and GacA system. RpoN in conjunction with HrpRS activates hrpL expression, and the genes targeted by HrpL have been identified (Chatterjee et al. 2002; Hendrickson et al. 2000). RpoN is also known to control COR synthesis (Smirnova et al. 2002). By contrast, those controlled by SalA in DC3000 are not yet known.
sion of hrp, dsp, and avr genes as well as other genes involved in bacterial virulence (Boch et al. 2002; Chatterjee et al. 2002; Collmer et al. 2000; Fouts et al. 2002; Frederick et al. 2001; Guttman et al. 2002; Mor et al. 2001; Wei and Beer 1995; Wei et al. 2000; Xiao et al. 1994). The effects of the GacA-deficient mutant on the elicitation of the HR and its reduced virulence in tomato and Arabidopsis are consistent with the effects on hrpL expression. DC3000 is clearly different from P. syringae pv. syringae B728a in this regard. We should recall that Willis and associates (1990) noted that deficiency of GacS and GacA in B728a apparently had no effect on the elicitation of HR in tobacco leaves but caused the loss of the ability to induce brown spot disease in bean. Our results from Northern blot analysis of the GacA+ mutant of B728a (BGACX) and its parent (Fig. 3B) now show that GacA deficiency does not affect the expression of hrpL and hrpZ, the latter encodes an effector and is known to be controlled by HrpL. This raises the question why the GacA-deficient mutant of B728a is avirulent but shows the HR. We tentatively attribute this to the effects of GacA-controlled SalA production on hrp, vir, and avr genes of P. syringae pv. syringae B728a (discussed below).

Willis and associates have shown that GacA positively regulates the production of SalA, a putative DNA-binding protein that controls toxin production and pathogenicity in P. syringae pv. syringae B728a (Kitten et al. 1998). They have also documented that the effect of SalA on pathogenicity is not due to its effect on toxin production (Lu et al. 2002). Thus, it is reasonable to assume that SalA function is dedicated toward the expression of genes for plant interaction in addition to toxinogenesis. We have shown that sala is expressed in DC3000, that the levels of salaDC3000 transcripts are higher in IM than in KB medium, and that GacA deficiency results in a severe reduction in salaDC3000 transcript levels. Extrapolating from the observations of Kitten and associates (1998) with P. syringae pv. syringae, we suggest that SalaDC3000 controls genes for pathogenicity in DC3000 as well and the GacA effect on virulence is due to the cumulative regulatory effects of SalaDC3000, HrpLDC3000, CorRDC3000, and CorSDC3000 (the effects on CorR and CorS are discussed below). In this regard, B728a and DC3000 behave differently. In B728a, sala but not hrpL expression is affected by GacA, contrasting with effects on both hrpL and sala in DC3000. A systematic search of B728a and DC3000 genes affected by HrpL deficiency and Sala deficiency would be most instructive.

Our data also establish for the first time that salaDC3000 expression is better stimulated in IM than in KB (Fig. 4D). While we do not have a clear understanding of the significance of this regulation, it is tempting to suggest that DC3000 produces effector proteins under physiologically challenging conditions and, to ensure this response, the bacterium utilizes regulators, i.e., HrpL and Sala, that are themselves stress-activated.

As opposed to Sala, much is now known about the functions of CorR and CorS in the context of coronatine biosynthesis (Bender et al. 1999; Peñaloza-vázquez and Bender 1998; Rangaswamy and Bender 2000; Smirnova et al. 2002; Wang et al. 1999). Thus, it is possible to predict that the regulation of these cor genes by GacA is significant in the contexts of production of chlorosis symptoms in susceptible interactions as well as elicitation of defense responses in incompatible hosts. As a virulence factor, coronatine induces changes in plant metabolic systems analogous to those induced by plant hormones. Coronatine bears remarkable structural similarities to methyl jasmonate and 12-oxo-phytodienoic acid, prompting the hypothesis that the toxin mimics the octadecanoid signaling molecules of higher plants. How GacA controls CorR and CorS remains an open question at this time. Again, our preliminary data suggest that the GacA-mediated regulation of rsmB and rsmZ may be responsible.

The growth rate of the GacADC3000 mutant in KB medium is very similar to that of DC3000 (Fig. 2). However, in minimal salts plus succinic acid medium, the mutant grows at a somewhat slower rate than the parent, although both attain the same cell density by the onset of stationary phase. Growth of the mutant in planta is reduced compared with that of the parent. These findings raise the possibility that GacADC3000 deficiency may lead to impaired growth in response to change in nutritional content of the apoplast fluid and such reduction in population may be responsible for reduced virulence. While we cannot yet formally rule out that GacADC3000 affects overall metabolic efficiency and, hence, virulence of DC3000, it is more likely that regulation of virulence effectors by GacADC3000 is actually responsible for disease production and the HR induction.

It has been documented that the GacS/GacA system regulates the quorum-sensing signal by positively controlling the biosynthesis of AHL in P. aeruginosa, P. aureofaciens, and P. syringae (Chancey et al. 1999; Dumenyo et al. 1998; Kendra et al. 2000; Kitten et al. 1998; Pessi and Haas 2001; Zhang and Pierson 2001). The quorum-sensing signal controls the HCN biosynthetic genes (hcnABC) in P. aeruginosa (Pessi and Haas 2001) and phenazine antibiotic biosynthesis genes pheXYFABCD in P. aureofaciens (Zhang and Pierson 2001). However, AHL has no effect on protease production in P. aureofaciens (Zhang and Pierson 2001) and various secondary metabolites, extracellular protease, and swarming motility in P. syringae pv. syringae (Dumenyo et al. 1998; Kinscherf and Willis 1999). We have shown that GacA is required for the production of AHL and expression of ah1 and ah1R in DC3000. However, we do not yet know if AHL controls the expression of genes involved in virulence of DC3000 or secondary metabolite production. We have begun searching for AHL-deficient mutants. Transcript profiles in DC3000, its AHL-deficient mutant, and GacA-deficient mutant should allow us to identify the AHL-dependent genes, AHL-independent genes, and the regulatory effects of GacADC3000 on these genes.

GacA positively regulates the expression of the sigma factor gene rpoS in P. syringae, P. fluorescens, E. coli, and Erwinia carotovora (Cui et al. 2001; Kinscherf and Willis 1999; Mukhopadhyay et al. 2000; Whistle et al. 1998). We document here that GacADC3000 controls transcript level of rpoS in DC3000 as well (Fig. 3A). We propose that GacS and GacA control RpoS production and various stress responses in DC3000 as a part of a regulatory cascade (Fig. 6). There is precedence in the literature for similar cascades controlling various traits in different bacteria. For example, in Azotobacter vinlandii (Castañeda et al. 2001), a similar cascade was found to control alginate production as well as gene expression during stationary phase. Likewise, Loper and associates determined that GacS and GacA played a critical role in the expression of rpoS in P. fluorescens and, consequently, had a marked effect on stress response and antifungal metabolite production (Whistle et al. 1998).

GacA is known to affect metabolites and proteins that are produced by bacteria during postexponential growth, presumably when bacteria are exposed to various stresses (Heeb and Haas 2001). How this is brought about is not yet known. However, the following possibilities merit consideration: i) the regulatory effects of GacA on rpoS, which is probably required to cope with various stress conditions; ii) the enhanced activity of GacA, which could depend upon the relative pool size of phosphorylated and dephosphorylated forms of GacA, assuming that the phosphorylated form functions as the transcriptional enhancer; and iii) the stimulation of GacA production during later growth stages and under stress conditions. Our data support the first and the last possibilities. The levels of gacA transcripts are higher during postexponential growth of
DC3000. More importantly, gacA transcript levels are much higher in IM than in KB. We should note that transfer from KB to IM represents a nutritional shift down as well as exposure to an acidic environment. Both these conditions may trigger stress response in DC3000, activating an acidic environment. Both these conditions may trigger stress to IM represents a nutritional shift down as well as exposure to DC3000. More importantly,

responses in DC3000, activating an acidic environment. Both these conditions may trigger stress to IM represents a nutritional shift down as well as exposure to DC3000. More importantly, loaded in each well. The RNA markers (0.28 to 6.58 kb) were denatured agarose gel, indicated by arrows in Figures 1 and 20 µg) were loaded in each lane as indicated in the figure captions. Representative pictures of ethidium bromide-staining of total RNAs (15 to 263 nt), amplified by PCR, using labeled gacAP1 and opposing primer gacAP2 (5′-TAGGTTGTAGCCGGATCAAT-3′) was end-labeled by 

Random-priming, according to the manufacturer’s instructions (Promega, Madison, WI, U.S.A.). The genomic library of DC3000 was transferred into a ClustalW program. Motifs search was done using the ExPasy Prositescan program, and default parameters were used.

Ahlh and ahlR genes of DC3000 were identified by searching the homolog of psm (AF234628.2) and psmR (AF234628.3) genes of P. syringae pv. maculicola in DC3000 genomic sequence. Prehybridization (6 h at 65°C) and hybridization (18 h at 65°C) were performed in hybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2× Denhardt’s, 0.1% sodium dodecyl sulfate [SDS], 100 µg of denatured salmon sperm DNA per ml). After hybridization, membranes were washed twice for 20 min each at 65°C in 2× SSC, 0.1% SDS and, then, for 30 min at 65°C in 0.5x SSC, 0.1% SDS and were examined by autoradiography with X-ray film (Kodak, Rochester, NY, U.S.A.).

S1 nuclease protection assay.

Primer gacAP1 (10 pmol) (5′-CATGGCGGTGTAGCCGGATCAAT-3′; complementary to the base position –84 to –60 from the translational start codon) was end-labeled by polynucleotide kinase and γ-32P[ATP]. The end-labeled probe was amplified by PCR, using labeled gacAP1 and opposing unlabeled primer gacAP2 (5′-TAGGTTGTAGCCGGATCAAT-3′) corresponding to the base positions -694 to -670 from the translational start codon) and pAKC1111 as template DNA. The conditions of PCR, hybridization, S1 nuclease digestion, and analysis of the products were as described by Liu and associates (1998).

Bioluminescence assay for AHL production.

DC3000 and its GacA.DC3000+ mutant AC811 were grown in KB medium at 28°C for 20 h, and culture supernatants were harvested for assays by using an E. coli-based bioassay system as described previously (Chatterjee et al. 1995). There is a linear relationship between the quantity of AHL and the emission of bioluminescence expressed as RLU per minute per milliliter.

Cloning of gacA.DC3000-

The genomic library of DC3000 was transferred into a GacA- mutant of Erwinia carotovora subsp. carotovora, AC5056, which does not produce extracellular protease (Cui et al. 2001). Two plasmids that could restore the extracellular protease production in AC5056 on nutrient gelatin agar were selected. Presence of gacA.DC3000 in those plasmids was confirmed by Southern blot analysis by using a PCR-amplified internal fragment of gacA.DC3000 as probe.

Construction of GacA.DC3000+ mutant.

To inactive the gacA.DC3000- Erwinia carotovora subsp. carotovora GacA+ strain AC5056 carrying the gacA.DC3000 plasmid pAKC1111 was mutagenized with mini-Tn5-Km’. The Tc′Km′ colonies that did not produce extracellular protease were selected, and insertion in GacA was confirmed by Southern blot analysis. The inactivated gacA.DC3000 plasmid pAKC1112 was transferred into DC3000. DC3000 carrying pAKC1112 was grown in KB plus Km at 28°C for 24 h, was diluted 1000-fold into fresh KB plus Km, and was incubated for an addi-
tional 24 h. Bacterial cells were then plated on KB plus Km agar and were patched on KB plus Km and KB plus Km and Tc. A Km·Tc colony was selected, and the absence of gacA RNA was confirmed by Northern blot analysis. The location of mini-Tn5-Km insertion was determined by sequence analysis, using pAKC1112 DNA as the template.

Swarming motility, pigment, and siderophore production tests.

For the swarming motility test, bacterial cells were inoculated onto SWM agar (0.4% agar) and were incubated at 28°C. Motility of the bacteria was visually examined.

Pigment production was tested on KB agar, and siderophore production was tested on CSA agar. Bacterial cells were inoculated on KB or CSA agar and were incubated at 28°C. Yellow pigment of bacteria was visually examined and the siderophore production was indicated by a clear zone around the colony.

HR and pathogenicity test.

Bacteria were grown on KB agar overnight at 28°C, and cells were suspended in water for HR or pathogenicity tests. The procedures for HR in tobacco leaves were previously published (Cui et al. 1996; Mukherjee et al. 1997). Young, fully expanded third and fourth leaves of 4- to 8-week-old Nicotiana tabacum cv. Samsun were used for the HR test. Bacterial cells (5 × 10^6 or 1 × 10^7 CFU/ml) were infiltrated into tobacco leaves as indicated in the figure captions. Pictures were taken 20 h after infiltration. For the pathogenicity test in Arabidopsis, leaves of 5-week-old plants (A. thaliana cv. Columbia) were infiltrated with bacterial cells (1 × 10^7 CFU/ml). Pictures were taken four days after inoculation. DC3000 strains were assessed for their ability to cause disease symptoms and multiply in plants by dipping tomato (Lycopersicon esculentum) cv. Moneymaker) plants into bacterial suspension, using pAKC1112 DNA as the template.

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LITERATURE CITED


**AUTHOR-RECOMMENDED INTERNET RESOURCES**

The Institute for Genomic Research (TIGR) web site: www.tigr.org
The Expert Protein analysis system (ExPASy) website: www.expasy.ch