Review

Regulatory Roles of the GacS/GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria

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The sensor kinase GacS and the response regulator GacA are members of a two-component system that is present in a wide variety of Gram-negative bacteria and has been studied mainly in enteric bacteria and fluorescent pseudomonads. The GacS/GacA system controls the production of secondary metabolites and extracellular enzymes involved in pathogenicity to plants and animals, biocontrol of soilborne plant diseases, ecological fitness, or tolerance to stress. A current model proposes that GacS senses a still-unknown signal and activates, via a phosphorelay mechanism, the GacA transcription regulator, which in turn triggers the expression of target genes. The GacS protein belongs to the unorthodox sensor kinases, characterized by an autophosphorylation, a receiver, and an output domain. The periplasmic loop domain of GacS is poorly conserved in diverse bacteria. Thus, a common signal interacting with this domain would be unexpected. Based on a comparison with the transcriptional regulator NarL, a secondary structure can be predicted for the GacA sensor kinases. Certain genes whose expression is regulated by the GacS/GacA system are regulated in parallel by the small RNA binding protein RsmA (CsrA) at a posttranscriptional level. It is suggested that the GacS/GacA system operates a switch between primary and secondary metabolism, with a major involvement of posttranscriptional control mechanisms.

Additional keywords: quorum sensing.

Two-component systems are widely distributed among bacteria and also occur in some eukaryotes. These regulatory systems help the organisms to adapt to different conditions and to colonize specific ecological niches in response to environmental signals. Typically, a two-component system consists of a sensor kinase and a cognate response regulator. Upon interaction with the signal(s), the sensor undergoes autophosphorylation and then activates the response regulator by phosphotransfer. The signals may be abiotic (e.g., pH, temperature, or osmolarity) or biotic; some signals are produced by a host, others are synthesized by the bacterial populations themselves. In the latter case, the signals may be produced in coordination with the cell density of the population and thereby ensure regulatory mechanisms commonly known as quorum sensing (Bassler 1999; Hoch 2000; Holden et al. 2000; Pirrung 1999; Rice et al. 1999; Stock et al. 2000). For a number of two-component systems, the signals are not yet known.

The GacS/GacA two-component system, whose functions and sequence conservation will be reviewed here, plays important roles in various Gram-negative bacteria. In plant- or animal-pathogenic bacteria, the GacS/GacA system can be required for virulence. In plant-beneficial bacteria, this system controls the expression of biocontrol factors that protect plants from pathogenic fungi.

Discovery of the GacS/GacA two-component system.

The sensor kinase GacS, initially called LemA, was first described in *Pseudomonas syringae* pv. *syringae* strain B728a as an essential factor for lesion manifestation by this pathogenic strain on bean leaves (Hrabak and Willis 1992; Kitten et al. 1998). Mutational inactivation of the *gacS* gene in this strain results in the loss of virulence and ecological fitness (Hirano et al. 1997; Hirano and Upper 2000; Hrabak and Willis 1992; Willis et al. 1990). The response regulator GacA was first described in *P. fluorescens* biocontrol strain CHA0 as a global activator of antibiotic and cyanide production (Laville et al. 1992). In this strain, GacA function is required for antifungal activity and ecological fitness (Laville et al. 1992; Natsch et al. 1994). The antifungal activity of strain CHA0 is brought about by a combination of several secondary metabolites (discussed below) that are essential to the biocontrol of fungal root pathogens (Haas et al. 2000). Since publication of the initial reports, GacS and GacA homologs have been characterized in a great variety of other Gram-negative bacteria (Table 1).

Evidence that GacS and GacA are partners of a two-component system was first obtained genetically in *P. syringae* pv. *syringae* (Rich et al. 1994) and subsequently confirmed for several other bacteria (Aarons et al. 2000; Altier et al. 2000b; Bull et al. 2001; Ligon et al. 2000; Pernestig et al. 2001; Whistler et al. 1998). Biochemical confirmation that GacA is the cognate response regulator of the GacS sensor has recently been provided by the demonstration of in vitro phosphotransfer from the GacS homolog BarA to the GacA homolog UvrY in *Escherichia coli* K-12 (Pernestig et al. 2001).

Important functions

of the GacS/GacA two-component system.

Close to 20 GacS and GacA homologs have been identified in enteric bacteria (*E. coli*, *Salmonella enterica*, and *Erwinia carotovora*), fluorescent pseudomonads, *Vibrio*, and *Azotobacter* (Table 1). In general, these homologs have been found because null mutations in the corresponding genes cause remarkable phenotypes. Two main properties of *gacS/gacA* mutants stand out: partial or complete loss of biocontrol ability in a group of plant-beneficial pseudomonads and markedly reduced virulence in plant- or animal-pathogenic bacteria (Table

1). A common denominator of these *gacS/gacA*-controlled phenotypes is the synthesis of extracellular products, i.e., enzymes and secondary metabolites (Altier et al. 2000b; Gaffney et al. 1994; Johnston et al. 1996; Laville et al. 1992; Whistler et al. 1998; Zhang and Normark 1996) (Table 1). The only microorganism of Table 1 that has neither pathogenic nor plant-beneficial effects, *E. coli* K-12, has been known for some time to have *gacS* and *gacA* homologs, usually designated *barA* and *uvrY*, respectively (Kahn and Ditta 1991; Moolenaar et al. 1987; Nagasawa et al. 1992; Sharma et al. 1986). Only recently has a phenotype of *barA* and *uvrY* mutations been re-

Table 1. Phenotypes controlled by the GacS/GacA two-component system in various Gram-negative bacteria

Strain	GacS homolog	GacA homolog	Comments on acronyms	Positive control of production of	Prominent effects of <i>gacS</i> or <i>gacA</i> mutations	References
<i>Pseudomonas aureofaciens</i> 30-84	LemA	GacA		<i>N</i> -Acyl-homoserine-lactones, phenazine antibiotics, cyanide, exoprotease		Chancey et al. 1999
<i>Pseudomonas chlororaphis</i> PCL1391	GacS			Phenazine antibiotics, chitinase, protease		Chin-A-Woeng 2000
<i>Pseudomonas fluorescens</i> BL915	LemA	GacA		Pyrrolnitrin, chitinase, cyanide, 2-hexyl-5-propyl-resorcinol, exoprotease	Reduced biocontrol and inhibition of <i>Rhizoctonia solani</i>	Gaffney et al. 1994 Ligon et al. 2000
<i>Pseudomonas fluorescens</i> CHA0 ^{*a}	GacS	GacA	Global activator of antibiotic and cyanide production	2,4-Diacetylphloroglucinol, pyoluteorin, cyanide, pyrrolnitrin, tryptophan side-chain oxidase, indole-3-acetic acid, exoprotease, phospholipase C	Reduced biocontrol activity in suppression of black root rot of tobacco and other plant diseases, reduced ecological fitness and survival in stationary phase	Bull et al. 2001 Duffy and D��fago 2000 Laville et al. 1992 Natsch et al. 1994 Oberh��nsli and D��fago 1991 Sacherer et al. 1994
<i>Pseudomonas fluorescens</i> F113	GacS	GacA		2,4-Diacetylphloroglucinol, cyanide, exoprotease		Aarons et al. 2000
<i>Pseudomonas fluorescens</i> Pf-5	GacS (=ApdA)	GacA	Antibiotic production	Pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, cyanide, exoprotease, tryptophan side-chain oxidase	Loss of inhibition of <i>Rhizoctonia solani</i> in culture, reduced stress resistance	Corbell and Loper 1995 Kraus and Loper 1992 Pfender et al. 1993 Whistler et al. 1998
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> AH2552 ^b	RpfA		Regulator of pathogenicity factors	Exoprotease, cellulase	Reduced pathogenicity on potato tubers	Frederick et al. 1997
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> SCC3193 ^b	ExpS	ExpA	Extracellular enzyme production	Pectate lyase, polygalacturonase, cellulase		Eriksson et al. 1998
<i>Pseudomonas marginalis</i> CY091	LemA			Pectate lyase, exoprotease, levan, pyoverdine	Loss of soft rot pathogenicity on potato tuber slices and detached pepper fruits	Liao et al. 1997
<i>Pseudomonas syringae</i> BR2R	LemA			Exoprotease, tabtoxin	Reduced plant pathogenicity on bean leaves	Barta et al. 1992
<i>Pseudomonas syringae</i> pv. <i>coronafaciens</i> Pc27R	LemA			Exoprotease, tabtoxin	Absence of chlorosis formation on oat leaves	Barta et al. 1992

(continued on next page)

^a In these organisms, the GacS/GacA system also controls negatively the production of (*) pyochelin, salicylic acid and a fluorescent compound (Duffy and Dfago 2000; Schmidli-Sacherer et al. 1997), (**) a fluorescent compound and motility (Grewal et al. 1995), or (***) flagella (Goodier and Ahmer 2001).

^b *Erwinia carotovora* subsp. *carotovora* has recently been renamed *Pectobacterium carotovorum* subsp. *carotovorum* (Hauben et al. 1998).

ported: both types of mutations lead to increased sensitivity to hydrogen peroxide stress, due to a reduced expression of catalase (Mukhopadhyay et al. 2000; Pernestig et al. 2001).

In a group of plant-beneficial, root-colonizing bacteria (*P. fluorescens* strains CHA0, Pf-5, BL915, and F113; *P. chlororaphis* strain PCL1391; and *P. aureofaciens* strain 30-84), the GacS/GacA system positively controls the expression of genes required for the synthesis of secondary metabolites with antimicrobial activity. These metabolites include 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, 2-hexyl-

5-propyl-resorcinol, and phenazine compounds (Table 1). Each of the *Pseudomonas* strains mentioned produces one or several of the above metabolites. Combined evidence from many independent studies indicates that bacterial production of these metabolites on plant roots is important for the protection of the colonized plants from a range of pathogenic fungi (Haas et al. 2000; Thomashow 1996). In the same bacteria, the GacS/GacA system also positively regulates the expression of genes coding for secreted enzymes, e.g., protease, phospholipase C, and chitinase. Although the roles of these enzymes in

Table 1. (continued from preceding page)

Strain	GacS homolog	GacA homolog	Comments on acronyms	Positive control of production of	Prominent effects of <i>gacS</i> or <i>gacA</i> mutations	References
<i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	GacS (=LemA)	GacA	Global activator sensor kinase; lesion manifestation	Exoprotease, syringomycin, syringolin, <i>N</i> -acyl-homoserine-lactones, alginate	Loss of brown spot disease formation on bean, loss of swarming capacity, reduced ecological fitness	Barta et al. 1993 Hrabak and Willis 1992 Hrabak and Willis 1993 Kitten et al. 1998 Rich et al. 1994 Wäspi et al. 1998 Willis et al. 1990
<i>Pseudomonas tolaasii</i> NCPPB1116**	PheN		Phenotypic variant	Tolaasin, exoprotease, colony morphology	Reduced brown blotch formation on <i>Agaricus bisporus</i> mushrooms	Grewal et al. 1995 Sinha et al. 2000
<i>Pseudomonas tolaasii</i> PT814	RtpA		Regulator of tolaasin production and other pleiotropic traits	Tolaasin, exoprotease, colony morphology	Loss of brown blotch on <i>Pleurotus ostreatus</i> fruiting bodies	Murata et al. 1998
<i>Pseudomonas viridiflava</i> PJ-08-6A and SF312A	RepA	RepB	Regulation of exoprotein production	Pectate lyase, exoprotease, fluorescent siderophores, alginate	Loss of soft rotting ability	Liao et al. 1994 Liao et al. 1996
<i>Pseudomonas aeruginosa</i> PAO1	GacS (=LemA)	GacA		Pyocyanin, cyanide, lipase, <i>N</i> -butyryl-homoserine-lactone	Reduced virulence in leukopenic mice	Parkins et al. 2001 Rahme et al. 1995 Reimann et al. 1997 Rich et al. 1994
<i>Pseudomonas aeruginosa</i> UCBPP-PA14	LemA (=GacS)	GacA			Reduced virulence in plants and animals, reduced biofilm formation	Parkins et al. 2001 Rahme et al. 1995 Rahme et al. 2000 Tan et al. 1999a Tan et al. 1999b
<i>Salmonella enterica</i> serovar <i>Typhimurium</i> ***	BarA	SirA	<i>Salmonella</i> invasion regulator	HilA-dependent regulation of pathogenicity island SPI1 and other pathogenicity factors	Reduction of swarming motility, reduction of gastroenteritis in bovine model, loss of type III secretion apparatus, loss of invasion endocytosis of epithelial cells	Altier et al. 2000b Johnston et al. 1996 Mukhopadhyay et al. 2000
<i>Vibrio cholerae</i>		VarA	Virulence associated regulator	Cholera toxin, toxin-coregulated pilus	Loss of autoagglutination, reduced infant mice colonization	Wong et al. 1998
<i>Escherichia coli</i> K12 and D517	BarA (=AirS)	UvrY	Bacterial adaptive responses; attachment and iron regulation sensor in strain D517	Siderophores and siderophore receptors; multicopy suppression of an <i>envZ</i> mutation	Increased sensitivity to H ₂ O ₂	Nagasawa et al. 1992 Zhang and Normark 1996
<i>Azotobacter vinelandii</i>	GacS			Alginate, polyhydroxybutyrate	Impaired encystment	Castañeda et al. 2000

biocontrol has not been investigated specifically in the strains listed in Table 1, it is known that exoprotease and chitinase can contribute to biocontrol activity in other bacterial isolates (Chernin et al. 1995; Dunne et al. 2000). Furthermore, tryptophan side-chain oxidase, an enzyme that initiates an indole-3-acetate biosynthetic pathway, is under GacS/GacA control in *P. fluorescens* strains CHA0 and Pf-5 and provides a convenient cytochemical marker for the function of this two-component system (Oberhänsli and Défago 1991; Voisard et al. 1994). Both *gacS* and *gacA* mutants of *P. fluorescens* strain CHA0 have altered colony morphology and motility behavior: colonies are larger and flatter on normal agar plates, whereas on semisolid agar, colonies show signs of altered motility by comparison with the wild-type strain (Duffy and Défago 2000; Goodier and Ahmer 2001), suggesting that the GacS/GacA system has an impact on cell surface components as well. Negative control exerted directly or indirectly by the GacS/GacA system has also been reported; in *P. fluorescens* strain CHA0, *gacS* and *gacA* mutations lead to the increased production of pyochelin and of a fluorescent compound, possibly pyoverdine (Duffy and Défago 2000; Schmidli-Sacherer et al. 1997).

The GacS/GacA system is required for lesion formation on leaves, but not for the hypersensitive reaction, in the plant pathogen *P. syringae* pv. *syringae* strain B728a. The toxin syringomycin, the extracellular polysaccharide alginate, and exoprotease, which are all controlled by GacS/GacA, are probably involved in pathogenicity, but individually, none of these traits are sufficient to cause a pathogenic interaction (Hirano and Upper 2000; Willis et al. 2001). In other leaf pathogens, such as *P. syringae* pv. *coronafaciens*, *P. viridiflava*, and *P. marginalis*, and in the mushroom pathogen *P. tolaasii*, the GacS/GacA system has a decisive role in various types of pathogenicity (Table 1). By contrast, the bean pathogen *P. syringae* pv. *phaseolicola* apparently does not require GacS/GacA function for lesion formation on bean (Rich et al. 1992).

P. syringae pv. *syringae* strain B301D-R elaborates a modified 12-membered peptide, syringolin, under GacS control. Syringolin induces acquired resistance in rice against the rice blast fungus *Pyricularia oryzae*; this elicitor is not directly toxic to the fungus but stimulates the expression of defense-related genes in rice, thereby preventing leaf necrosis (Wäspi et al. 1998).

Azotobacter vinelandii, a nitrogen-fixing, nonsymbiotic soil bacterium, differentiates into desiccation-resistant resting cells called cysts. In this bacterium, encystment as well as alginate production are under GacS control (Castañeda et al. 2000).

Soft rot disease caused by *Erwinia carotovora* subsp. *carotovora* on crop plants (e.g., potato) is characterized by extensive maceration of infected tissue. Maceration is the result of plant cell wall-degrading enzymes, such as cellulase, pectate lyase, and protease, that are secreted by the bacterium. Mutations in the *gacS* (= *expS*, *rpfA*) or the *gacA* (= *expA*) gene lead to a pleiotropic loss of the extracellular enzymes and virulence (Cui et al. 2001; Eriksson et al. 1998; Frederick et al. 1997). Harpin, a secreted protein that elicits a hypersensitive reaction on tobacco leaves, is also produced under GacS/GacA control in *Erwinia* spp. (Cui et al. 2001).

The ubiquitous bacterium and opportunistic pathogen *P. aeruginosa*, when infiltrated at relatively high cell densities into leaves of *Arabidopsis* or lettuce, causes extensive tissue damage manifested by soft rot and chlorosis. In this infiltra-

tion assay, *gacA* mutants cause much less damage (Rahme et al. 1995, 1997, 2000). Interestingly, in several animal models—burnt mice, fast-killing of the nematode *Caenorhabditis elegans*, and injected wax moth larvae—*gacA* or *gacS* mutants of *P. aeruginosa* are also much less virulent (Jander et al. 2000; Tan et al. 1999b), suggesting that the GacS/GacA system regulates factors that are required for colonization of tissues in all hosts tested (Mahajan-Miklos et al. 2000). *P. aeruginosa* virulence factors under positive GacA control include the phenazine compound pyocyanine, cyanide, and lipase (Pessi and Haas 2001; Reimmann et al. 1997).

In *Salmonella enterica* serovar *Typhimurium*, the GacA homolog SirA, with its cognate sensor BarA (a GacS homolog), positively regulates type III secretion and invasion functions (Ahmer et al. 1999; Goodier and Ahmer 2001; Johnston et al. 1996; Lucas et al. 2000). In a bovine ileal loop model of gastroenteritis, *sirA* mutants are strongly attenuated (Ahmer et al. 1999). Invasion and other virulence factors depend on the central transcriptional regulator HilA encoded by *Salmonella* pathogenicity island 1 (SPI1); SirA and other regulators act positively on the expression of HilA (Cotter and DiRita 2000; Lucas and Lee 2001).

Cholera toxin and the toxin-coregulated pili depend on the GacA homolog VarA for expression in *Vibrio cholerae*. As a consequence, a *varA* mutant has reduced colonization ability in infant mice (Wong et al. 1998).

In summary, there is a great variety of GacA-dependent phenotypes in beneficial or pathogenic bacteria (Table 1). As shown by several detailed kinetic studies, these phenotypes are mostly expressed when bacterial cells grown in batch culture (thus in the absence of a host organism) are in the transition from exponential to stationary phase (Blumer et al. 1999; Eriksson et al. 1998; Johnston et al. 1996; Reimmann et al. 1997). In other words, the GacS/GacA system can be active in the absence of host cells, and this activity is low when cells essentially devote their metabolic energy to rapid exponential growth, when they are present at low density, or both. This observation points to a possible link between quorum-sensing mechanisms and GacS/GacA control. At the end of this review, we will discuss this point further.

Essential structural features of the GacS/GacA system.

GacS belongs to a class of unorthodox histidine sensor kinases that carry a phosphoryl transmitter, a receiver, and a histidine phosphotransfer (Hpt) output domain (Perraud et al. 2000). Similar modular structures occur in other sensor kinases, especially in ArcB, which senses changes in redox potential and oxygen tension in *E. coli* (Georgellis et al. 1997, 1998, 1999; Kwon et al. 2000), and in BvgS, which controls virulence of *Bordetella pertussis* (Perraud et al. 2000). Based on sequence similarities, several functional domains can be postulated in GacS (Fig. 1). In the N-terminal part, the sensing domain, there are two potential transmembrane segments (Fig. 1, hatched boxes) separated by a periplasmic loop. This periplasmic loop is a common feature of many two-component histidine kinases (Dutta et al. 1999; Stock et al. 2000). Adjacent to the second transmembrane domain, two amphipathic sequences (Fig. 1, open boxes) are found in the linker domain of GacS. These sequences are proposed to interact with each other when an environmental signal triggers the activation of the protein, inducing a conformational change of the cytoplas-

mic C-terminal part and thereby favoring autophosphorylation (Robinson et al. 2000; Williams and Stewart 1999). Next to this amphipathic linker region lies the primary transmitter domain, with a conserved autophosphorylatable histidine residue (Fig. 1, H₁). Alternating β -sheets and α -helices occur in this domain, which is also important for dimerization of sensor kinases (Dutta et al. 1999). After autophosphorylation, the phosphoryl group is transferred to an aspartate residue (Fig. 1, D) of the primary receiver domain in GacS and subsequently to a histidine residue (Fig. 1, H₂) belonging to the carboxy-terminal Hpt domain in GacS. The Hpt domain acts as a secondary transmitter by transferring the phosphoryl group to a conserved aspartate of response regulator GacA (Fig. 1) (Pernestig et al. 2001). Dephosphorylation and concomitant inactivation of the GacS/GacA system may be accomplished in a similar but opposite way, ending with the release of inorganic phosphate in the cytoplasm, as shown for ArcB (Georgellis et al. 1998, 1999). Phosphorylation causes dimerization of FixJ of *Sinorhizobium meliloti*, a response regulator related to GacA, and dimerization is required for high-affinity binding of FixJ to target DNA (Da Re et al. 1999). Thus, both GacS and GacA might be active as dimers. GacA, like other response regulators, contains a helix-turn-helix motif involved in DNA binding. The DNA sequence recognized by GacA and the (regula-

tory) genes directly controlled by GacA are unknown. It is assumed that major effects of the GacS/GacA system on target genes are indirect (Fig. 1).

Conserved domains in GacS homologs.

Taking the GacS protein sequence from *P. fluorescens* strain CHA0 as the query (GenBank accession no. AF246292), homologs were searched in National Center for Biotechnology Information (NCBI) databases using the BLAST algorithm (Altschul et al. 1990). Preliminary and finished sequences from genome-sequencing projects were also screened for GacS homologs. Incomplete sequences as well as sensor kinases showing an end-to-end similarity below 42% (using GAP; Needleman and Wunsch 1970) were discarded. This means that some sensor kinases reported to be distantly related to GacS (e.g., *Xanthomonas campestris* RpfC, 80 kDa; Slater et al. 2000), which do not meet these arbitrary criteria, were not taken into account. A GacS alignment using PILEUP (Feng and Doolittle 1987) identifies, not surprisingly, three groups of high similarity: the pseudomonads, the enterics, and *Vibrio/Shewanella* (Fig. 2). Inside the pseudomonads, the GacS proteins of *P. fluorescens* strains CHA0 and Pf-5, *P. chlororaphis*, *P. tolaasii*, *P. viridiflava*, and *P. syringae* belong to a subgroup, with the GacS homologs of *P. aeruginosa* and *P. putida*

Fig. 1. Model of the signal transduction pathway mediated by the GacS/GacA two-component system. Autophosphorylation of GacS at the first conserved histidine residue (H₁) is triggered by unknown signal(s). A phosphoryl group is transferred by a phosphorelay mechanism to conserved aspartate and histidine residues in the domains D and H₂, respectively, of GacS. A conserved aspartate in GacA then acts as a phosphoryl acceptor. The phosphorylated response regulator GacA activates (or represses) the transcription of unidentified regulatory elements, which in turn control the expression of target genes, at least in part, at a posttranscriptional level (described in text). In a few microorganisms, autoinducers (*N*-acyl homoserine lactones) are signals under GacS/GacA control. N and C in lozenge, N and C terminus, respectively; and HTH, helix-turn-helix motif.

at some distance (Fig. 2). This pattern of clustering coincides with 16S rRNA data, which show close relationships between *P. fluorescens* strain CHA0, *P. chlororaphis*, and *P. syringae* (Ramette et al. 2001).

The 18 GacS sensor kinase sequences were then aligned using CLUSTAL W (Thompson et al. 1994) and further analyzed with PLOTSIMILARITY (Wisconsin Package version 10.1; Genetics Computer Group, Madison, WI, U.S.A.), a program used to determine the similarity average in windows of 10 amino acid residues (Fig. 3). The transmitter, receiver, and Hpt output domains are conserved clearly above the average of similarity of the aligned sequences. A fourth element, the linker, also shows conservation above average (Fig. 3). An especially high degree of conservation is seen surrounding the site of autophosphorylation of the first conserved histidine, corresponding to the H-box. It has been proposed that the region comprising the H-box and the adjacent linker domain may contain determinants that impart recognition and interaction specificity for the cognate response regulator (Wright and Kadner 2001). In the GacS receiver domain, three regions surrounding the conserved aspartate-aspartate (DD), the phosphorylatable aspartate (D), and the lysine (K) appear clearly on the plot. Finally, in the output domain, two peaks of residue

conservation are noticeable: a region previously unreported in the N-terminal part of the output domain and a region surrounding the third phosphorylation site (Fig. 3). Three regions of GacS show a relatively low degree of conservation (Fig. 3): the periplasmic loop including both transmembrane domains (residues 1 to 200), the region between the transmitter and the receiver (residues 520 to 680), and the short sequence between the receiver and the output domains (residues 800 to 840). It is interesting to note that the periplasmic loop does not have a high degree of conservation. If there were a signal common to all GacS sensor kinases (and such a signal remains to be identified), then the periplasmic loop would not appear to be a likely candidate for interaction with the signal. Rather, the periplasmic domain could have a modulatory function, in response to different environmental signals in different bacterial species.

Conserved features of GacA homologs.

GacA homologs were identified in NCBI databases using the BLAST algorithm, with the wild-type GacA protein sequence from *P. fluorescens* strain CHA0 as the query (Bull et al. 2001; Reimann et al. 1997). Hits showing an end-to-end identity below 40% were discarded. This threshold was chosen to exclude the *Ralstonia solanacearum* VsrD protein,

Fig. 2. Dendrograms showing the order of pairwise alignments of GacS and GacA sequences obtained by PILEUP. GenBank accession numbers or other references to the sequences are indicated in parentheses: Avi, *Azotobacter vinelandii* UW 136 (GacS: AF197912); Eco, *Escherichia coli* K-12 (BarA: D10888; UvrY: M24615); Pae, *Pseudomonas aeruginosa* PAO1 (GacS: AF030352; GacA: U27988); Pau, *P. aureofaciens* 30-84 (GacA: AF115381); Eca1, *Erwinia carotovora* subsp. *carotovora* AH2 (RpfA: U26023); Eca2, *Erwinia carotovora* subsp. *carotovora* SCC3193 (ExpS: Y13670; ExpA: X95564); Pch, *P. chlororaphis* O6 (GacS: AF192795); Pfl1, *P. fluorescens* CHA0 (GacS: AF246292; GacA: M80913); Pfl2, *P. fluorescens* Pf-5 (GacS: U30858; GacA: AF065156); Pfl3, *P. fluorescens* BL915 (GacA: L29642); Ppu, *P. putida* KT2440 (TIGR); Psy, *P. syringae* pv. *syringae* B728a (GacS: M80477; GacA: U09767); Pto1, *P. tolaasii* NCPPB1116S (PheN: U25692/U95300); Pto2, *P. tolaasii* PT814 (RtpA: AB002529); Pvi, *P. viridiflava* PJ-08-6A (RepA: L30101; RepB: L30102); Spa, *Salmonella paratyphi* A (GSC-WU); Spu, *Shewanella putrefaciens* MR-1 (TIGR); Sty1, *Salmonella enterica* serovar *Typhimurium* ATCC 14028 (BarA: AF171069; SirA: U67869); Sty2, *Salmonella typhi* CT18 (Sanger); Vch1, *Vibrio cholerae* O395 (VarA: AF071105); Vch2, *Vibrio cholerae* biovar *El Tor* (XxxS: AE004315); Xfa, *Xylella fastidiosa* 9a5c (XxxA: XF2608); and Ype, *Yersinia pestis* CO-92 biovar *orientalis* (Sanger). Sequences that have not yet been named are designated XxxS or XxxA. Some sequences are from genome-sequencing projects of The Institute for Genomic Research (TIGR), the Genome Sequencing Center at Washington University (GSC-WU), or the Sanger Center (Sanger).

which has been considered to be a GacA homolog (Huang et al. 1995) but whose corresponding sensor kinase, VsrA, is unrelated to GacS. A dendrogram showing the order of pairwise alignments of GacA sequences (Fig. 2) places the GacA proteins of four biocontrol strains of fluorescent pseudomonads (CHA0, Pf-5, BL915, and 30-84) into one cluster, close to the GacA proteins of *P. syringae* and *P. viridiflava* and somewhat removed from those of *P. aeruginosa* and *P. putida*. As in the GacS dendrogram, the GacA homologs of enteric and other bacteria (*Shewanella* and *Xylella*) are placed outside the *Pseudomonas* group.

An alignment of the 16 GacA sequences using CLUSTAL W (Thompson et al. 1994) (Fig. 4) allows secondary structure predictions based on a comparison with structurally characterized response regulators, in particular NarL of *E. coli* and FixJ of *Sinorhizobium meliloti* (Baikalov et al. 1996; Birck et al. 1999; Stock et al. 2000). Essential structural features of the active site in the N-terminal receiver domain (residues 1 to 148), as determined for NarL and FixJ, include the phosphorylatable Asp-54 residue and its conserved contacts with Asp-8/Asp-9, Thr(Ser)-82, and Lys-104 (Fig. 4). In the C-terminal DNA-binding domain (residues 149 to end), a helix-turn-helix motif occurs that is expected to determine the (currently unknown) DNA recognition specificity of GacA.

Conserved neighborhood of *gacA* and *uvrC*.

The genes encoding a sensor kinase–response regulator pair are often next to each other. The *gacS* and *gacA* genes of the organisms shown in Figure 2, by contrast, are never immediate neighbors. However, another kind of neighborhood is

striking. In 15 microorganisms examined, the *gacA* gene is located upstream of a homolog of the *E. coli* *uvrC* gene (Fig. 5). The only apparent exception is the putative *gacA* gene of *Xylella fastidiosa*, which lies upstream of an RNase E gene (Fig. 5). The UvrC protein is part of the UvrABC complex that mediates nucleotide excision repair; UvrC makes incisions upstream and downstream of UV-damaged DNA (Tang et al. 2001). In *E. coli* K-12, the *gacA* homolog *uvrY* does not have a function in excision repair (Moolenaar et al. 1987). In this species, there is experimental evidence showing that *uvrY* and the downstream *uvrC* gene belong to a common transcriptional unit. Moreover, a second *uvrC* promoter is located in the 3' coding region of *uvrY* (Moolenaar et al. 1987; Sharma et al. 1986). In *P. fluorescens* (Laville et al. 1992), *P. aeruginosa* (Reimann et al. 1997), *Salmonella typhimurium* (Ahmer et al. 1998), and *Erwinia carotovora* (Eriksson et al. 1998) polar mutations in *gacA* drastically reduce UV tolerance, indicating that *gacA* and *uvrC* are cotranscribed also in these bacteria. However, there is no evidence that the GacA protein makes a contribution to UV repair. The question then arises as to whether the *uvrC* gene has a role in the GacS/GacA regulation system. In one study on *Yersinia enterocolitica*, it has been shown that a *uvrC* mutation reduces the expression of invasins, a primary invasion factor. However, it is uncertain whether this phenotype might have been accompanied by a reduction in the expression of the *gacA* homolog located immediately upstream (Badger and Miller 1998).

Under laboratory conditions, spontaneous *gacS* or *gacA* pleiotropic mutants have been observed to appear at high frequencies in cultures of *P. fluorescens* (Gaffney et al. 1994;

Fig. 3. Schematic representation of the GacS sensor kinase and plot representing the average of similarity among 18 GacS homologs in a multiple sequence alignment. PLOTSIMILARITY was performed on the sequences aligned with CLUSTAL W (Thompson et al. 1994). Regions showing a score above the average are well-conserved sequences that are analyzed in windows of 10 amino acids. By analogy with other, better-characterized sensor kinases, such as ArcB and BvgS, the following functional domains can be distinguished in GacS: TM1/TM2, transmembrane segments; PL, periplasmic loop; ASI/ASII, amphipathic sequences (Williams and Stewart 1999); Q, Q-rich sequence (Wootton and Drummond 1989); H, H-box (transmitter phosphorylation site); N/G1/F/G2, N, G1, F, and G2 boxes, respectively (Parkinson and Kofoed 1992); and C and N in lozenge, C and N terminus, respectively.

Laville et al. 1992), *P. syringae* (Rich and Willis 1997; Rich et al. 1994), or *P. tolaasii* (Grewal et al. 1995). In the latter organism, loss of GacS function is caused by a reversible insertion (internal duplication) (Sinha et al. 2000). Loss of GacS or GacA function due to deletion or point mutations has been observed in *P. fluorescens* (Bull et al. 2001; Gaffney et al. 1994); however, there is no evidence for reversibility of these mutations, e.g., via a "phase-variation" mechanism. There are indications suggesting that nutrient-rich media, with a relatively high osmotic pressure, may favor, at least at some stages of growth, the mutants over the wild type (Bull et al. 2001; Duffy and Dfago 2000). When media are amended with Zn²⁺, Cu²⁺, or Co²⁺ ions at subinhibitory concentrations, the selective advantage of the *gacS* and *gacA* mutants over the wild type is abolished (Duffy and Dfago 2000). Since the mutants are not hypersensitive to transition elements, these amendments are thought to select for the wild type in an indirect way. Whether or not this selective force is related to DNA-damage repair or to some other stress response remains to be determined.

The GacS/GacA regulatory cascade contains posttranscriptional control elements.

The response regulator GacA activates, or represses, the expression of regulatory genes, probably at the transcriptional level (Fig. 1). A detailed analysis in *P. fluorescens* strain CHA0 has shown that GacA does not interact directly with the promoter of the GacA-controlled target genes *hcnABC* (Blumer and Haas 2000a; Blumer et al. 1999). In fact, when an *hcnA*'-*lacZ* translational fusion is expressed from the *tac* promoter, this construct remains fully dependent on the GacS/GacA system and no part of the *hcnA* promoter appears to be responsive to GacA (Blumer and Haas 2000a; Blumer et al. 1999). A region surrounding the *hcnA* ribosome-binding site, about 11 nucleotides in length, is instrumental for GacA control (Blumer et al. 1999; K. Starke and D. Haas, unpublished data). Exactly the same region is also involved in the repression by the

RNA-binding protein RsmA, suggesting that RsmA may be a downstream element in the GacS/GacA regulatory cascade (Blumer et al. 1999). RsmA (for regulator of secondary metabolism) is a small RNA-binding protein that is conserved in many Gram-negative as well as in some Gram-positive bacteria (Cui et al. 2001; Romeo 1998; Whittle et al. 1999). RsmA of *Erwinia carotovora* and its homolog CsrA of *E. coli* act as mRNA decay factors, probably by interacting with mRNAs near the ribosome-binding sites of target genes (Mukherjee et al. 1996). In *Erwinia carotovora*, an *rsmA*-negative mutant is hypervirulent and overproduces extracellular lytic enzymes (Chatterjee et al. 1995). In *E. coli* K-12, CsrA has been characterized mostly as a posttranscriptional repressor of glycogen biosynthesis (Romeo 1998). However, a *csrA* mutant has diverse pleiotropic phenotypes related to central carbon metabolism, e.g., a defect in acetate utilization (Wei et al. 2000). The *csrA* gene of *Salmonella enterica* serovar Typhimurium is required for invasion of epithelial cells; however, a *csrA* mutation has less drastic effects than have *barA* (*gacS*) and *sirA* (*gacA*) mutations (Altier et al. 2000a). In *P. fluorescens* strain CHA0, overexpression of RsmA represses the expression of GacA-controlled target genes including *hcnA*, *aprA* (for exoprotease), and *phlA* (for 2,4-diacetylphloroglucinol biosynthesis) (Blumer et al. 1999; C. Gigot-Bonnefoy and D. Haas, unpublished data), whereas an *rsmA* mutation partially suppresses the exoproduct defect of a *gacS* null mutation (Blumer et al. 1999).

The repressive activity of RsmA (CsrA) is antagonized by a small, nontranslated, regulatory RNA designated RsmB in *Erwinia carotovora* or CsrB in *E. coli* and *Salmonella enterica*. Multiple stem-loop structures, with loops displaying typical ribosome-binding site patterns (AGGA), are the most salient feature of these regulatory RNAs (Altier et al. 2000b; Liu et al. 1997, 1998; Romeo 1998). CsrB overproduction suppresses a *barA* defect in *Salmonella enterica* (Altier et al. 2000b). Similarly, in *P. fluorescens* strain F113, overexpression of the regulatory RNA PrrB partially restores secondary

Sty1SirA	TGT AAT GCG GAG ACG TTA ACA AGC CAG <u>TG</u> AGT GAA ATA TTT GAC GCA AAG->	uvrC
Sty2XxxA	TGT AAT GCG GAG ACG TTA ACA AGC CAG <u>TG</u> AGT GAA ATA TTT GAC GCA AAG->	uvrC
EcoUvrY	TGT AAT GCG GAG ACA TTA TCA AGT CAG <u>TG</u> AGT GAT CAG TTT GAC GCA AAA->	uvrC
YpeXxxA	TTT AAT GCG GAG ACG TTA TCA AAT AGT G ACT GAT CTT TTT GAT TAT AAA->	uvrC
Eca2ExpA	TTT ACC GCG GAG ACA TTG TTA AGT AGT G AGT GAG AGT TTC GAT GCT TCG->	uvrC
Vch1VarA	TTA GAT ACT GAG AAG TTA TA	GTG TCG ACA CAG TTT GAT TCT GCC->
PsyGacA	GTT GAT GCC AGC GCC <u>TGA</u> AC	ATG ACC CAG ACA TT- GAT CCA GGT->
PviRepB	GTG GAC GCC AGC GCC <u>TGA</u> AC	ATG ACC CAG ACC TTT GAT CCA AGT->
PauGacA	GTC GAT GCC AGC GCC <u>TGA</u> CA	ATG ACC GAC CCG TTT GAT CCC AGT->
Pfl3GacA	GTC GAT GCC AGT GCC <u>TGA</u> CA	ATG ACC GAC CCG TTT GAT CCC AGT->
Pfl1GacA	GTT GAT GCC AGC CTC <u>TGA</u> A	ATG ACC GAA CAG TTT GAT CCC AGT->
Pfl2GacA	GTT GAT GCC AGC CTC <u>TGA</u> A	ATG ACC GAA CAG TTT GAT CCC AGT->
PpuXxxA	GTT GAC GCA AGC CTG <u>TAA</u> AACCTC	ATG TCC CAA GTC TTT GAT GCC AGC->
PaeGacA	GTC GAT GCC GCC AGC <u>TAG</u>	ATG AGC GCC GTT TTC GAC GCG AGC->
SpuXxxA	TTA GAT GCT GGA CAT <u>TTT</u> <u>TAA</u> TCTATAGCATTAAATTCCTGCTTATATAGTGTGTTA->	70 bp uvrC
XfaXxxA	AGT ACT CCG GAG AAA TAT AGT TTA GAT AAA ACG ATC <u>TGA</u> TTCGAAATCCAGAT->	rne (?)

Fig. 5. Alignment of the distal part of *gacA* homologs in Gram-negative bacteria. The *gacA* stop codons are underlined. Putative *uvrC* start codons are indicated in boldface. In *Xylella fastidiosa* (Xfa), an open reading frame showing homology to the *Escherichia coli* *rne* gene lies downstream of the *gacA* homolog. Eco, *Escherichia coli* K-12; Pae, *Pseudomonas aeruginosa* PAO1; Pau, *P. aureofaciens* 30-84; Eca2, *Erwinia carotovora* subsp. *carotovora* SCC3193; Pfl1, *P. fluorescens* CHA0; Pfl2, *P. fluorescens* Pf-5; Pfl3, *P. fluorescens* BL915; Ppu, *P. putida* KT2440; Psy, *P. syringae* pv. *syringae* B728a; Pvi, *P. viridiflava* PJ-08-6A; Spu, *Shewanella putrefaciens* MR-1; Sty1, *Salmonella enterica* serovar Typhimurium ATCC 14028; Sty2, *Salmonella typhi* CT18; Vch1, *Vibrio cholerae* O395; Xfa, *Xylella fastidiosa* 9a5c; and Ype, *Yersinia pestis* CO-92 biovar *orientalis*.

metabolite production to *gacS* and *gacA* mutants (Aarons et al. 2000). Like RsmB/CsrB, PrrB contains multiple stem-loop structures, with a consensus AGGA sequence placed in the loops (Aarons et al. 2000). In *P. fluorescens* strain CHA0, a structurally related RNA (termed RsmZ) has been found as an RNA being noncovalently bound to RsmA protein (S. Heeb and D. Haas, unpublished data). The GacS/GacA system positively controls the expression of RsmB-like regulatory RNAs. This has been shown in *Erwinia carotovora* (Cui et al. 2001; Hyytiäinen et al. 2001) and *P. fluorescens* strain CHA0 (S. Heeb and D. Haas, unpublished data).

These findings in various Gram-negative bacteria lead to a model that undoubtedly needs to be refined as more and more elements of the GacS/GacA cascade are being discovered. Some essential parts in this model include the following. (i) During transition from exponential to stationary phase, i.e., in the idiophase, the GacS/GacA system is activated, most probably by the phosphorelay depicted in Figure 2. The signals activating and deactivating the system are not known. (ii) Activated GacA has a positive effect, directly or indirectly, on regulatory elements involved in exoproduct formation, such as RsmB and related regulatory RNAs. (iii) These regulatory RNAs sequester the RNA-binding protein RsmA and thereby relieve translational repression of target mRNAs. However, posttranscriptional control exerted by RsmA and RsmB can account only for part of the effects steered by the GacS/GacA system, implying that further regulatory elements must be involved.

What are these additional elements? In *P. syringae* pv. *syringae* strain B728a and in *P. fluorescens* strain CHA0, overexpression of the *infC* operon can partially suppress a *gacS* or *gacA* mutation (Blumer and Haas 2000b; Kitten and Willis 1996). The *infC* operon encodes the translation initiation factor IF3 and the ribosomal proteins L35 and L20. The intact operon carried by a multicopy plasmid has the highest suppressive effect; deletion analysis primarily points to a role of the two 50S subunit ribosomal proteins (Blumer and Haas 2000b; Kitten and Willis 1996). The overexpressed *infC* operon relieves RsmA-mediated posttranscriptional repression of a target gene (*hcnA*) in *P. fluorescens* strain CHA0, suggesting that some product(s) of the *infC* operon can compete with RsmA (Blumer and Haas 2000b).

The expression of the stationary-phase sigma factor σ^S (the *rpoS* product) is regulated positively by GacS/GacA in *P. fluorescens* strain Pf-5 (Whistler et al. 1998) and *E. coli* (Mukhopadhyay et al. 2000). Thus, the manifestation of some GacS/GacA-dependent phenotypes may be determined by RpoS. However, regulation of *rpoS* expression is extremely complex (Loewen et al. 1998) and, at least in *P. fluorescens*, *rpoS* and *gacS/gacA* mutants have dissimilar phenotypes (Sarniguet et al. 1995; Whistler et al. 1998). Currently, the interactions between the RpoS and the GacS/GacA regulons are poorly understood.

In three bacterial species, *P. aeruginosa*, *P. syringae*, and *P. aureofaciens*, the GacS/GacA system acts on the quorum-sensing machinery by positively regulating the synthesis of N-acyl-homoserine lactones (AHLs) (Chancey et al. 1999; Kitten et al. 1998; Pessi and Haas 2001; Reimann et al. 1997). AHLs are also known as autoinducers. These signal molecules are produced by a variety of Gram-negative bacteria and mediate gene expression as a function of population density (Fuqua

and Greenberg 1998; Williams et al. 2000). In the three AHL-producing organisms mentioned, GacA-dependent formation of exoproducts proceeds via at least two pathways. One involves stimulation of AHL production and AHL-activated transcriptional control of target genes. The other pathway does not depend on AHLs and operates on the same target genes at a posttranscriptional level (Chancey et al. 1999; Pessi and Haas 2001). It remains to be seen whether GacA-dependent stimulation of AHL synthesis involves the same regulatory elements (e.g., RsmA) as does the AHL-independent pathway.

In conclusion, many beneficial or pathogenic interactions of Gram-negative bacteria with their host organisms (plants as well as animals) are critically influenced by the GacS/GacA two-component system. The current challenges are to discover the signal molecule(s) specific to the GacS sensor and to identify additional components in this complex regulatory pathway.

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F. S. L. Brinkman, E. L. A. Macfarlane, P. Warrenner, and R. E. W. Hancock (Evolutionary relationships among virulence-associated histidine kinases. 2001. Infect. Immun. 69:5207-5211.) point out the occurrence of sensor kinases related to GacS in *Streptomyces coelicolor* and in fungi.

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