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Signal transduction in plant-beneficial rhizobacteria with biocontrol properties

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Abstract

Biological control of root pathogens – mostly fungi – can be achieved by the introduction of selected bacterial inoculants acting as 'biopesticides'. Successful inoculants have been identified among Gram-negative and Gram-positive bacteria, often belonging to *Pseudomonas* spp. and *Bacillus* spp., respectively. Biocontrol activity of a model rhizobacterium, *P. fluorescens* CHA0, depends to a considerable extent on the synthesis of extracellular antimicrobial secondary metabolites and exoenzymes, thought to antagonize the pathogenicity of a variety of phytopathogenic fungi. The regulation of exoproduct formation in *P. fluorescens* (as well as in other bacteria) depends essentially on the GacS/GacA two-component system, which activates a largely unknown signal transduction pathway. However, recent evidence indicates that GacS/GacA control has a major impact on target gene expression at a post-transcriptional level, involving an mRNA target sequence (typically near the ribosome binding site), two RNA binding proteins (designated RsmA and RsmE), and a regulatory RNA (RsmZ) capable of binding RsmA. The expression and activity of the regulatory system is stimulated by at least one low-molecular-weight signal. The timing and specificity of this switch from primary to secondary metabolism are essential for effective biocontrol.

Abbreviations: AHL – *N*-acyl-homoserine lactone; ISR – induced systemic resistance; PCA – phenazine carboxylic acid; PHL – 2,4-diacetylphloroglucinol; PLT – pyoluteorin; RBS – ribosome binding site; SAR – systemic acquired resistance; TSO – tryptophan side-chain oxidase

Introduction

Plant roots provide an attractive, nutrient-rich environment to a large number of soil microorganisms. The zone in the vicinity of the root surface, the rhizosphere, typically contains 10 to 100 times more microorganisms per gram than does bulk soil. The rhizosphere microorganisms, in turn, can have a decisive influence on plant health. Root pathogens, mostly fungi, can penetrate the root tissue and cause extensive damage to crop plants. Certain plant-beneficial microorganisms (especially strains belonging to *Pseudomonas, Bacillus, Streptomyces* and *Trichoderma* spp.) are known to antagonize the effects of these pathogens in the rhizosphere (Cook 1993; Thomashow & Weller 1995; Keel & Défago 1997; Emmert & Handelsman 1999; Ellis et al. 2000; Whipps 2001). From ecological as well as from economical points of view, it is particularly rewarding to analyze those situations in which the effects of the beneficial (biocontrol) microorganisms outweigh those of the pathogens. Such can be the case in natural *disease-suppressive soils*, of which several examples have been studied in various parts of the world. In addition, a certain level of disease-suppressiveness can also be induced by crop practice: in fields where wheat is cultivated year after year, take-all disease (caused by *Gaeumannomyces graminis* var. *tritici*) tends to become less prevalent after 3 to 5 years. This *take-all decline* phenomenon, like natural disease-suppressiveness, depends to a large extent, but not solely, on the activities of biocontrol microorganisms (Cook 1993).

In his book 'Biological control of microbial plant pathogens', R. Campbell (1989) concluded: "It is a common story for biological control of soil diseases, and take-all in particular, there seems to be something useful going on, but it cannot be really defined, quantified and repeated successfully". How has this sceptical outlook evolved since the time when it was written? Raaijmakers & Weller (2001) put it this way: "In evaluating the last decade of research on biological control (of soil-borne plant pathogens), it is clear that most biocontrol agents, including strains of antibiotic-producing *Pseudomonas* spp., are still too variable in their performance to be successfully used as a common practice in agriculture and horticulture. This inconsistency has been attributed to a number of factors, including the variable expression of genes involved in disease suppression and poor root colonization by the applied biocontrol agent."

In the absence of a major commercial breakthrough in agriculture, biocontrol research has tended to focus on a few selected biocontrol strains with outstanding properties and on the use of microcosms. The main advantages of microcosms are that environmental parameters, plant growth, and disease pressure can be controlled and standardized. Biocontrol activity of introduced strains can then be defined, quantified and reproduced – as postulated by Campbell (1989). In this reductionist approach, molecular genetic techniques (construction of defined mutations, complementation, use of reporter genes to measure biocontrol gene expression) have played a major role and have helped to unravel important biocontrol traits, e.g. those pertaining to biocontrol strains of fluorescent pseudomonads.

Biocontrol traits in biocontrol rhizobacteria

Several recent reviews (Haas et al. 2000; Bloemberg & Lugtenberg 2001; Walsh et al. 2001) have presented essentially three important properties of effective biocontrol strains (with emphasis on fluorescent pseudomonads): Rhizosphere competence, antibiosis, and stimulation of plant defense (Table 1). Rhizosphere competence includes bacterial traits that allow aggressive root colonization down to the tip (such as flagella, pili, lipopolysaccharide, the *sss* recombinase assumed to play a role in surface variation, etc.) (De Weger et al. 1987; Simons et al. 1996; Dekkers et al. 1998a, b, 2000; Lugtenberg & Dekkers 1999; Chin-A-Woeng et al. 2000; Lugtenberg et al. 2001; Benizri et al. 2001; Turnbull et al. 2001; Espinosa-Urgel et al. 2002). Rhizosphere competence also implies that the biocontrol bacteria are well adapted to the utilization of root exudate compounds (carboxylic acids, sugars, certain amino acids) and possess specific, siderophore-mediated iron uptake systems (Raaijmakers et al. 1995; Simons et al. 1997; Loper & Henkels 1999; Lugtenberg & Dekkers 1999; Lugtenberg et al. 1999; Rainey 1999; Kuiper et al. 2001; Lugtenberg et al. 2001; Mirleau et al. 2001). However, these nutritional characteristics are not unique to biocontrol strains and can also be found in deleterious or neutral rhizosphere microorganisms.

Antibiosis as a biocontrol mechanism has received most attention in the last decade of research on plant-beneficial rhizobacteria (Thomashow & Weller 1995; Keel & Défago 1997; Bender et al. 1999; Haas et al. 2000). The finding that certain bacterial secondary metabolites having antifungal properties (Table 1 lists some of them) determine to a large extent the biocontrol performance of many rhizobacteria, has led, on occasion, to a misnomer: such bacteria have sometimes been classified as 'biopesticides', as if they acted to kill the pathogens in the rhizosphere. However, to our knowledge, no biocontrol strain has ever been shown to kill, let alone to eradicate a root pathogen *in situ*. Several antimicrobial metabolites produced by biocontrol strains – phenazine carboxylic acids (PCA), pyoluteorin (PLT), 2,4-diacetylphloroglucinol (PHL) – have been detected and quantified in rhizosphere samples (Thomashow et al. 1990; Keel et al. 1992; Maurhofer et al. 1995; Bonsall et al. 1997; Raaijmakers et al. 1999). The amounts usually found (typically in the micromolar range) are insufficient to massively kill pathogenic and other microorganisms in the rhizosphere, but may locally protect the producer bacteria in their ecological niche, i.e., the microcolony. We therefore feel that the term 'biopesticide' should be reserved to biological control agents that kill. For example, the *Bacillus thuringiensis* endotoxin is a true biopesticide: it kills lepidopteran pests.

Several lines of evidence have established an important role of diffusible or volatile antibiotic compounds in the biological control of soil-borne diseases. (i) Mutants of *Pseudomonas* spp. defective in the production of specific antibiotic compounds (e.g. PCA, PHL, PLT, hydrogen cyanide [HCN], etc.) have lost part of their plant-protective ability (Thomashow & Weller 1988; Voisard et al. 1989; Fenton et al. 1992; Keel et al. 1992; Maurhofer et al. 1994a; Kraus & Loper 1995; Rodriguez & Pfender 1997; Chin-A-

Table 1. Important traits of biocontrol rhizobacteria

Trait	Examples of bacterial mechanisms/molecules involved	References
Rhizosphere competence		
- Root colonization	Flagella, lipopolysaccharide, *sss* recombinase, . . .	De Weger et al. 1987; Simons et al. 1996; Dekkers et al. 1998a; 1998b; 2000; Benizri et al. 2001; Turnbull et al. 2001
- Competition for exudates and Fe^{3+}	Catabolic versatility, siderophores	Raaijmakers et al. 1995; Simons et al. 1997; Loper & Henkels 1999; Lugtenberg et al. 1999; Kuiper et al. 2001; Mirleau et al. 2001
- Endophytic colonization	?	Duiff et al. 1997; Hallmann et al. 1997; Troxler et al. 1997; M'piga et al. 1997
Antibiosis		
- Production of diffusible components	Phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, . . .	Thomashow & Weller 1988; Fenton et al. 1992; Keel et al. 1992; Maurhofer et al. 1994b; Pierson III et al. 1995; Hammer et al. 1997; Rodriguez & Pfender 1997; Chin-A-Woeng et al. 1998; Kirner et al. 1998; Mavrodi et al. 1998; Bangera & Thomashow 1999; Hammer et al. 1999; Nowak-Thompson et al. 1999; Delany et al. 2000; Schnider-Keel et al. 2000; Tambong & Höfte 2001
- Production of volatile compounds	HCN	Voisard et al. 1989; Laville et al. 1998; Blumer & Haas 2000
Stimulation of plant defense		
- Induction of ISR[a] via the jasmonate-ethylene pathway	Lipopolysaccharide, siderophores (?)	Hoffland et al. 1995; Leeman et al. 1995; 1996; van Wees et al. 1997 Pieterse et al. 1998; Knoester et al. 1999; Pieterse & van Loon 1999; Ton et al. 2002
- Induction of SAR[b] via the salicylate pathway	Salicylate	Maurhofer et al. 1994a; 1998; DeMeyer & Höfte 1997; van Loon et al. 1998; DeMeyer et al. 1999

[a]ISR, induced systemic resistance.
[b]SAR, systemic acquired resistance.

Woeng et al. 1998; Tambong & Höfte 2001). (ii) Genetically engineered, enhanced antibiotic expression in biocontrol strains can result in improved biocontrol activity (Maurhofer et al. 1992; 1995; Timms-Wilson et al. 2000; Chin-A-Woeng et al. 2001; Delany et al. 2001). However, some compounds such as PHL and PLT, at high concentrations, can also be phytotoxic (Keel et al. 1992; Maurhofer et al. 1992; 1995). Therefore, care has to be taken to avoid these phytotoxic effects in genetically modified bacteria. (iii) Expression of antibiotic biosynthesis genes of fluorescent pseudomonads occurs in the rhizosphere and can be monitored by the use of reporter genes (Georgakopolous et al. 1994; Kraus & Loper 1995; Wood et al. 1997; Chin-A-Woeng et al. 1998; Pierson III et al. 1998; Notz et al. 2001; Seveno et al. 2001). The level and timing of antibiotic gene expression depends on bacterial signaling pathways (see below) as well as on environmental stimuli (Shanahan et al. 1992; Slininger & Sheawilbur 1995; Duffy & Défago 1999; Schnider-Keel et al. 2000; Notz et al. 2001). In particular, plant root exudates can have a strong impact on the expression of these genes. For instance, the PHL biosynthetic genes of *P. fluorescens* are more strongly expressed on corn and wheat roots than on bean and cucumber roots (Notz et al. 2001).

Resistance to pathogens can be induced in plants, essentially via two signaling pathways. In the first of these, termed systemic acquired resistance (SAR), salicylate is a key signal molecule (Reymond & Farmer 1998; van Loon et al. 1998). Upon interaction with bacterial, viral or fungal necrotizing pathogens, many plants respond by enhanced salicylate production, locally at the infection site as well as systemically. The question then arises as to whether root-colonizing, salicylate producing bacteria can activate the SAR pathway. In several plant-pathogen systems (e.g., tobacco – tobacco necrosis virus; bean – *Botrytis cinerea*), salicylate supplied exogenously by fluorescent pseudomonads has afforded some protection against the pathogens (Maurhofer et al. 1994a; 1998; DeMeyer & Höfte 1997; DeMeyer et al. 1999). However, the degree of protection is below that obtained with some functional analogs of salicylate used to elicit SAR, i.e. 2,6-dichloro-isonicotinic acid or benzothiadiazole.

The second, well-characterized defense pathway in plants involves jasmonate as a signal (Reymond & Farmer 1998). This mechanism can be activated by certain non-pathogenic rhizobacteria and has been designated induced systemic resistance (ISR) (van Loon et al. 1998; Pieterse & van Loon 1999; Ton et al. 2002). Although several bacterial traits (e.g., siderophores and lipopolysaccharides) have been proposed to trigger ISR (Hoffland et al. 1995; Leeman et al. 1995; 1996; van Wees et al. 1997), there is at present no compelling evidence for a specific ISR signal produced by bacteria. By contrast, in *Arabidopsis thaliana* several mutations have been described which do not express ISR upon exposure to selected rhizobacteria and which are blocked in the jasmonate-ethylene signal transduction pathway (Pieterse et al. 1998; Knoester et al. 1999; Pieterse & van Loon 1999). Interestingly, best biocontrol results are obtained by the combined activation of SAR and ISR (van Wees et al. 2000).

Some plant-beneficial bacteria may owe their biocontrol activity to predation on pathogens or to degradation of virulence factors produced by pathogens. However, in the case of fluorescent pseudomonads, such biocontrol mechanisms (if they exist) have not been documented in molecular detail.

Biocontrol of root diseases critically depends on the population densities of the beneficial microorganisms in the rhizosphere and typically occurs above threshold levels of 10^5 to 10^6 CFU/g of root for biocontrol pseudomonads (Raaijmakers & Weller 1998; Raaijmakers et al. 1999; Paulitz 2000). This observation implies, on the one hand, that under natural conditions biocontrol bacteria represent, at most, a few percent of all rhizosphere microorganisms. On the other hand, local cell densities of biocontrol bacteria in microcolonies (Chin-A-Woeng et al. 1997; Lugtenberg & Dekkers 1999; Bloemberg et al. 2000; Lugtenberg et al. 2001) are probably of paramount importance in that the synthesis of antimicrobial compounds is regulated by cell density-dependent signaling.

Importance of cell–cell signaling in biocontrol bacteria

Bacterial populations can coordinate certain activities in concert with cell densities. This phenomenon, which is commonly known as quorum sensing, relies on the accumulation of extracellular signal compounds (also termed pheromones), which are produced by the bacteria themselves and which, above certain threshold concentrations, modulate expression of target genes (Bassler 1999; De Kievit & Iglewski 2000; Williams et al. 2000; Fuqua et al. 2001; Whitehead et al. 2001). Quorum sensing-regulated functions

Table 2. Strains of fluorescent pseudomonads in which the GacS/GacA two-component system has a demonstrated role in biocontrol

Strain	Products under GacS/GacA control	References
P. aureofaciens 30-84	Phenazine antibiotics, HCN, exoprotease, *N*-acyl-homoserine lactones	Chancey et al. 1999
P. chlororaphis PCL1391	Phenazine antibiotics, exoprotease, chitinase, *N*-acyl-homoserine lactones	Chin-A-Woeng 2000
P. fluorescens BL915	Pyrrolnitrin, HCN, 2-hexyl-5-propyl-resorcinol, exoprotease	Gaffney et al. 1994; Ligon et al. 2000
P. fluorescens CHA0	PHL, PLT, HCN, pyrrolnitrin, phospholipase C, exoprotease, tryptophan side chain oxidase (=TSO)	Laville et al. 1992; Sacherer et al. 1994; Duffy & Défago 2000; Bull et al. 2001
P. fluorescens F113	PHL, HCN, exoprotease	Aarons et al. 2000; Sánchez-Contreras et al. 2002
P. fluorescens Pf-5	PHL, PLT, HCN, pyrrolnitrin, TSO	Corbell & Loper 1995; Whistler et al. 1998

typically help the bacterial populations to maintain themselves in ecological niches, e.g. by producing extracellular antimicrobial metabolites or enzymes. These extracellular products can be biocontrol factors or virulence factors, depending on the nature of the microbe–host interaction. In Gram-positive bacteria, peptide pheromones are common, whereas in a range of Gram-negative bacteria quorum sensing signaling can be provided by *N*-acyl-homoserine lactones (AHLs) (Bassler 1999; Williams et al. 2000). However, in many bacteria the cell density-related signals have not yet been identified chemically (Bassler 1999; Schauder et al. 2001).

Most AHLs are the products of an enzyme family, named LuxI after the prototype enzyme of *Vibrio fischeri* (De Kievit & Iglewski 2000; Fuqua et al. 2001). These enzymes charge an acyl chain from acyl carrier protein onto homoserine lactone recruited from *S*-adenosyl-methionine (Parsek et al. 1999). In two biocontrol strains, *P. aureofaciens* 30-84 and *P. chlororaphis* PCL1391, the LuxI homolog PhzI produces *N*-hexanoyl-homoserine lactone (HHL) as the major product (Pierson III et al. 1998; Chin-A-Woeng et al. 2001). HHL is recognized by the transcriptional activator PhzR (a member of the LuxR family of AHL-activated regulatory proteins), which is required for expression of phenazine antibiotics in both biocontrol strains (Pierson III et al. 1998; Chin-A-Woeng et al. 2001). Thus, in both strains, PhzI function is important for biocontrol. In *P. fluorescens* F113, *N*-(3-hydroxy-7-cis-tetradecenoyl)-homoserine lactone has been detected, the product of a novel AHL synthase (HdtS), which is not in the LuxI family (Laue et al. 2000). The biological functions of this novel AHL are not yet known. In other well-characterized biocontrol strains, e.g., *P. fluorescens* Pf-5 and *P. fluorescens* CHA0, there is evidence for quorum signals which are unrelated to AHLs but whose chemical structures await elucidation (Heeb et al. 2002; unpublished results of our laboratory).

The expression of the quorum sensing regulatory pair PhzR-HHL in the biocontrol strains *P. chlororaphis* PCL1391 and *P. aureofaciens* 30-84 depends on the two-component system GacS/GacA (Chancey et al. 1999; Chin-A-Woeng 2000). Similarly, in the opportunistic pathogen *P. aeruginosa* GacS/GacA positively regulates the quorum sensing machinery (Reimmann et al. 1997; Pessi & Haas 2001). This widely conserved two-component system consists of the sensor kinase GacS (equipped with a primary autophosphorylation/transmitter domain, a receiver domain, and a C-terminal secondary transmitter termed Hpt) and the response regulator GacA (Figure 1; Heeb & Haas 2001). Phosphotransfer from the GacS homolog BarA to the GacA homolog UvrY of *Escherichia coli* has been demonstrated *in vitro* (Pernestig et al. 2001). The signal(s) activating GacS are unknown. Interestingly, GacS/GacA control also operates in bacteria in which AHL signals have not been detected, e.g. in the biocontrol strains Pf-5, CHA0 and BL915 of *P. fluorescens* (Whistler et al. 1998; Haas et al. 2000; Ligon et al. 2000). In each case, the GacS/GacA system is essential for the expression of extracellular biocontrol factors (Table 2) and *gacS/gacA* mutants are strongly impaired in biocontrol activity in several plant-pathogen systems.

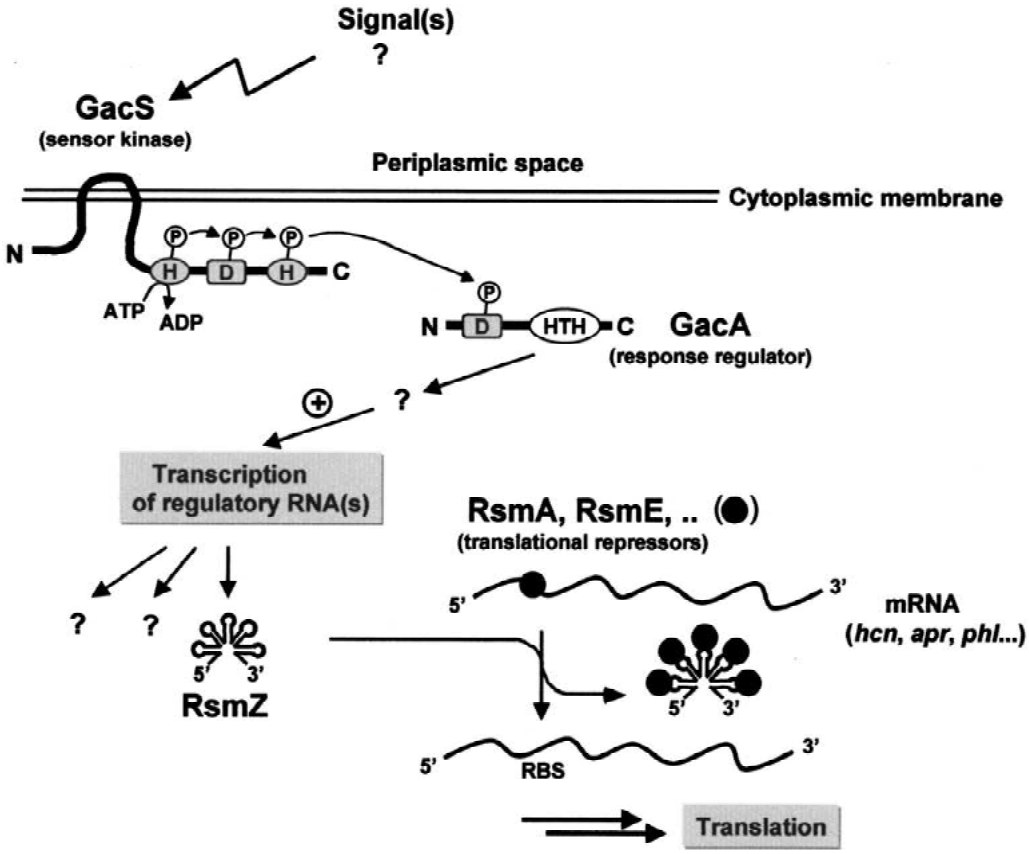

Figure 1. Proposed pathway of signal transduction involving the two-component system GacS/GacA in *P. fluorescens* CHA0. Activation of the sensor kinase GacS by unknown signals triggers a phosphorelay (H [His]→ D [Asp]→ H [His] in GacS→ D [Asp] in GacA), activating the response regulator GacA. Phosphorylated GacA positively controls transcription of regulatory RNA(s) such as RsmZ which sequester translational repressors like RsmA, rendering the RBS of target mRNAs accessible for translation; *hcn, apr*, and *phl* denote biosynthetic genes for HCN, alkaline protease, and PHL, respectively. Note that induction of *rsmZ* transcription by GacA may be indirect. Whether RsmZ sequesters the RsmA homolog RsmE has not yet been determined.

How does the GacS/GacA system regulate biocontrol activity? At present, the genes directly controlled by the transcriptional regulator GacA are unknown. From a limited genetic analysis, it appears that the structural genes encoding biocontrol factors are not subject themselves to transcriptional control by GacA (Heeb & Haas 2001). Rather, recent evidence obtained from *P. fluorescens* CHA0 suggests that GacA control of biocontrol functions is exerted essentially at a post-transcriptional level (Blumer et al. 1999; Haas et al. 2000). This control involves several elements of which only a few have been elucidated by mutational analysis. In *P. fluorescens* as well as in the plant pathogen *Erwinia carotovora*, GacA positively controls the expression of an untranslated regulatory RNA, termed PrrB (in strain F113) (Aarons et al. 2000), RsmZ (in strain CHA0) (Heeb et al. 2002) or RsmB (in *E. carotovora*) (Cui et al. 2001; Hyytiäinen et al. 2001). This RNA has a poorly conserved nucleotide sequence, but a characteristic secondary structure consisting of a number of stem-loop elements, with ribosome binding site (RBS) motifs in the loops (Figure 1). In Gram-negative bacteria, RsmB and its homologs are known to sequester a small RNA-binding protein, termed RsmA (CsrA), thereby preventing mRNA decay (Romeo 1998; Cui et al. 2001; Ma et al. 2001). It is believed that RsmA binds to target mRNAs at or near the RBS. Whether RsmA being a small protein of about 7 kDa has some mRNA recognition specificity itself, remains to be investigated. In strain CHA0, overexpression of RsmZ or mutational inactivation of RsmA cause derepression of the synthesis of biocontrol factors (Blumer et al. 1999; Heeb et al. 2002). Conversely, overexpression of RsmA results in repres-

sion of the synthesis of these factors (Blumer et al. 1999). These findings lead to a simplified model (Figure 1), according to which the GacS/GacA system, towards the end of exponential growth, upregulates the production of regulatory RNAs. These regulators then may relieve translational repression of target mRNAs by RsmA (Figure 1).

Clearly, a number of regulatory elements are still missing from this hypothetical scheme. For instance, an *rsmZ*-negative mutant of strain CHA0 is only weakly affected in the production of extracellular metabolites (Heeb et al. 2002). Hence, it is possible that other GacA-controlled regulatory RNAs may exist. Similarly, RsmA may not be the sole RNA-binding protein involved. In our recent experiments, we have obtained evidence for a second, structurally related RNA-binding protein, RsmE (unpublished results of our laboratory). Several point mutations in the RBS region preceding the *hcnABC* cluster (encoding HCN synthase) abolish or alter regulation by GacA, RsmA and RsmE (Blumer et al. 1999; unpublished results of our laboratory), suggesting that both RsmA and RsmE are downstream elements of the GacS/GacA signal transduction pathway. It will be interesting to see where exactly the AHL and non-AHL signals interact in this regulatory cascade in various biocontrol strains.

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