ORIGINAL ARTICLE

Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0

Bérénice Humair, Nicolas González[1], Dimitris Mossialos[2], Cornelia Reimmann and Dieter Haas

Département de Microbiologie Fondamentale, Université de Lausanne, Lausanne, Switzerland

In the plant-beneficial, root-colonizing strain *Pseudomonas fluorescens* CHA0, the Gac/Rsm signal transduction pathway positively regulates the synthesis of biocontrol factors (mostly antifungal secondary metabolites) and contributes to oxidative stress response via the stress sigma factor RpoS. The backbone of this pathway consists of the GacS/GacA two-component system, which activates the expression of three small regulatory RNAs (RsmX, RsmY, RsmZ) and thereby counters translational repression exerted by the RsmA and RsmE proteins on target mRNAs encoding biocontrol factors. We found that the expression of typical biocontrol factors, that is, antibiotic compounds and hydrogen cyanide (involving the *phlA* and *hcnA* genes), was significantly lower at 35 °C than at 30 °C. The expression of the *rpoS* gene was affected in parallel. This temperature control depended on RetS, a sensor kinase acting as an antagonist of the GacS/GacA system. An additional sensor kinase, LadS, which activated the GacS/GacA system, apparently did not contribute to thermosensitivity. Mutations in *gacS* or *gacA* were epistatic to (that is, they overruled) mutations in *retS* or *ladS* for expression of the small RNAs RsmXYZ. These data are consistent with a model according to which RetS–GacS and LadS–GacS interactions shape the output of the Gac/Rsm pathway and the environmental temperature influences the RetS–GacS interaction in *P. fluorescens* CHA0.

Introduction

Pseudomonas fluorescens CHA0, a model biocontrol bacterium originally isolated from a tobacco field in Switzerland, is an effective antagonist of plant-pathogenic fungi and nematodes that cause root diseases. Like other biocontrol bacteria, strain CHA0 colonizes the rhizosphere of important crop plants and produces several antibiotic compounds and lytic exoenzymes, which are important biocontrol factors accounting for suppression of root diseases (Haas and Défago, 2005; Mark *et al.*, 2006; Loper *et al.*, 2007; Mercado-Blanco and Bakker, 2007). The expression of biocontrol factors depends on the Gac/

Rsm signal transduction pathway (Laville *et al.*, 1992; Zuber *et al.*, 2003). In this regulatory cascade, the GacS/GacA two-component system activates the transcription of three small RNAs (sRNAs) termed RsmX, RsmY and RsmZ when cells reach high population densities (Heeb *et al.*, 2002; Valverde *et al.*, 2003; Kay *et al.*, 2005). These sRNAs avidly bind two sRNA-binding proteins belonging to the RsmA/CsrA family, named RsmA and RsmE (Reimmann *et al.*, 2005). In this way, translational repression exerted by these proteins can be relieved and target mRNAs become accessible to ribosomes for translation (Valverde *et al.*, 2004; Lapouge *et al.*, 2007, 2008). Typical target genes are, on the one hand, genes involved in biocontrol such as *hcnA* (for hydrogen cyanide (HCN) synthesis) and *phlA* (for synthesis of the antifungal metabolite 2, 4-diacetylphloroglucinol (DAPG)) and, on the other hand, the *rpoS* gene encoding the stress and stationary phase sigma factor σ^{38}, which is involved in the response of *P. fluorescens* to oxidative stress (Blumer *et al.*, 1999; Heeb *et al.*, 2005; Kay *et al.*, 2005). Thus, mutants affected in the GacS/GacA two-component system produce dramatically reduced amounts of secondary metabolites and are

Correspondence: D Haas, Département de Microbiologie Fondamentale, Biophore, Université de Lausanne, CH-1015 Lausanne, Switzerland.
E-mail: Dieter.Haas@unil.ch
[1]Current address: Institut de Microbiologie, Université de Lausanne, Rue du Bugnon 48, CH-1011 Lausanne, Switzerland.
[2]Current address: Department of Biochemistry and Biotechnology, University of Thessaly, Ploutonos 26 and Eolou Street, GR-41221 Larissa, Greece.

more sensitive to hydrogen peroxide, compared to the wild type (Laville *et al.*, 1992; Zuber *et al.*, 2003; Heeb *et al.*, 2005).

In pseudomonads, the activity of the Gac/Rsm pathway is regulated by autoinducing signal molecules whose chemical structures are unknown. These signals are thought to activate phosphorylation of the unorthodox GacS sensor (Heeb *et al.*, 2002; Zuber *et al.*, 2003; Dubuis *et al.*, 2007). Phosphorylated GacS then activates the response regulator GacA via a phosphorelay mechanism, for which experimental evidence has been obtained in *Pseudomonas aeruginosa* (Goodman *et al.*, 2009). In this organism, two additional sensors provide input into the Gac/Rsm pathway. These hybrid sensors termed RetS (for regulator of exopolysaccharide and type III secretion) and LadS (for lost adherence) were discovered in screens for mutants that form increased or decreased amounts of biofilm polysaccharides. It has been shown that RetS inhibits and LadS activates the activity of the Gac/Rsm pathway (Goodman *et al.*, 2004; Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). There is evidence that both RetS and LadS physically interact with GacS (Goodman *et al.*, 2009; Workentine *et al.*, 2009). However, the mechanisms by which these sensors communicate with one another and thereby determine the output of the system are not known. Moreover, the function of the Gac/Rsm pathway can be influenced by environmental cues. For instance, in *Escherichia coli* low pH values inhibit the activity of the BarA/UvrY proteins, which are GacS/GacA homologues (Mondragón *et al.*, 2006).

In various biocontrol strains of *P. fluorescens* it has been observed that incubation temperatures around 35 °C have a negative effect on biocontrol efficacy *in vivo* and on the expression of biocontrol factors such as DAPG and phenazine-1-carboxylic acid *in vitro* (Shanahan *et al.*, 1992; Slininger and Shea-Wilbur, 1995; Schmidt *et al.*, 2004). We found that in strain CHA0, too, the production of antibiotic compounds and HCN was reduced at 35 °C, by comparison with the production at 30 °C. This suggested to us that some component of the Gac/Rsm pathway might be sensitive to elevated temperature. The aim of this study, therefore, was to find this component. This led us to examine the roles of the RetS and LadS sensors in strain CHA0; RetS was found to be involved in temperature control. Using a genetic approach, we show that mutations in *gacS* or *gacA* override the effects of mutations in *retS* or *ladS*. These findings are consistent with GacS interacting directly with RetS and LadS.

Materials and methods

Bacterial strains, plasmids and growth conditions
The bacterial strains and plasmids are listed in Table 1. Strains of *E. coli* and *P. fluorescens* were grown in nutrient yeast broth (NYB) with shaking or on nutrient agar plates (Stanisich and Holloway, 1972). When required, antibiotics were added at the following concentrations: ampicillin (Ap), 100 µg ml^{-1} (only for *E. coli*); gentamicin (Gm), 10 µg ml^{-1}; kanamycin (Km), 25 µg ml^{-1}; tetracycline (Tc), 25 µg ml^{-1} (for *E. coli*) or 125 µg ml^{-1} (for *P. fluorescens*). In the mobilization of suicide plasmids (pME3087 derivatives) from *E. coli* to *P. fluorescens*, chloramphenicol (Cm) at 10 µg ml^{-1} and Tc were used to select for the recipient having integrated the suicide plasmid. Enrichment for Tc-sensitive strains, from which the suicide plasmid had been excised, was performed by exposing cells growing in NYB (at approximately 10^8 cells per ml) to Tc (20 µg ml^{-1}) for 1 h, followed by the addition of cycloserine (1.6 mg ml^{-1}) and further incubation for 5 h. For detection of *lacZ* constructs, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to plates at a final concentration of 0.02%. Routine incubation temperatures were 30 °C for *P. fluorescens* and 37 °C for *E. coli*. Alternatively, *P. fluorescens* was grown at 35 °C to test the temperature sensitivity of the Gac/Rsm signal transduction pathway and to improve its capacity to accept heterologous DNA originating from *E. coli*.

DNA manipulation
Small-scale plasmid extractions were done with the QIAprep Spin Miniprep Kit (Qiagen, Basel, Switzerland), whereas large-scale preparations were performed with the Jetstar kit (Genomed GmbH, Basel, Switzerland). Chromosomal DNA from *P. fluorescens* was prepared as previously described (Gamper *et al.*, 1992). DNA manipulations were carried out by standard techniques (Sambrook and Russell, 2001). DNA fragments were purified from agarose gels with the MinElute or QIAquick Gel extraction kits (Qiagen), depending on the fragment size. Electroporation of bacterial cells with plamid DNA was done as described (Farinha and Kropinsky, 1990). Conditions for amplifying PCR fragments were as follows: 200 ng of genomic DNA was dissolved in a final volume of 20 µl containing 200 µM of each of the four dNTPs, 20 pmol of each of two primers, 2 U of GoTaq polymerase (Promega, Catalys, Wallisellen, Switzerland) and 1 × GoTaq buffer (Promega). The PCR cycle was 2 min at 95 °C, 30 × (45 s at 95 °C, 45 s at 50–60 °C (depending on the G + C content and length of the primers), 0.5-2 min at 72 °C (depending on the length of the amplicon)) and a final elongation step of 5 min at 72 °C. The reaction products were purified on an agarose gel and the purified fragments were sequenced with an automatic sequencer.

Plasmid and strain constructions
These were facilitated by the fact that strain CHA0 is very similar to the completely sequenced strain Pf-5

Table 1 Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Genotype, phenotype or relevant characteristics	Reference or origin
Pseudomonas fluorescens strains		
CHA0	Wild type	Voisard *et al.* (1994)
CHA19	Δ*gacS*	Zuber *et al.* (2003)
CHA19.8	Δ*gacS* mini-Tn*7*Gm^r *gacS*Δ*76*	Zuber *et al.* (2003)
CHA89	*gacA*::Km^r	Laville *et al.* (1992)
CHA1202	Δ*retS*	This study
CHA1204	Δ*ladS*	This study
CHA1301	Δ*retS* Δ*gacS*	This study
CHA1302	Δ*ladS* Δ*gacS*	This study
CHA1303	*gacA*::Km^r Δ*retS*	This study
CHA1304	*gacA*:: Km^r Δ*ladS*	This study
CHA1305	Δ*retS* Δ*ladS*	This study
Plasmids		
pBluescript-II KS	Cloning vector, ColE1-replicon; Ap^r	Stratagene
pME497	Mobilizing plasmid; Ap^r	Voisard *et al.* (1994)
pME3087	Suicide vector; ColE1-replicon, IncP-1, Mob; Tc^r	Voisard *et al.* (1994)
pME3280a	Mini-Tn*7* gene delivery vector; Gm^r	Zuber *et al.* (2003)
pME6015	Vector for translational '*lacZ* fusion; Tc^r	Schnider-Keel *et al.* (2000)
pME6091	Transcriptional *rsmZ-lacZ* fusion; Tc^r	Heeb *et al.* (2002)
pME6182	Mini-Tn*7* gene delivery vector based on pME3280a, *Hin*dIII-*Sma*I-*Kpn*I-*Nco*I-*Sph*I MCS, ColE1 replicon; Gm^r Ap^r	This study
pME6259	Translational *phlA'-'lacZ* fusion, under *phlA* promoter; Tc^r	Schnider-Keel *et al.* (2000)
pME6355	Translational *rpoS'-'lacZ* fusion, under *rpoS* promoter; Tc^r	Heeb *et al.* (2005)
pME6530	Translational *hcnA'-'lacZ* fusion, under p*tac*, Tc^r	Blumer *et al.* (1999)
pME6702	Translational *phlA'-'lacZ* fusion, under p*tac*, Tc^r	Heeb *et al.* (2005)
pME6916	Transcriptional *rsmY-lacZ* fusion; Tc^r	Valverde *et al.* 2003)
pME7317	Transcriptional *rsmX-lacZ* fusion; Tc^r	Kay *et al.* (2005)
pME7698	pME6182 with a 3.5-kb fragment containing *rsmX-lacZ*; Gm^r Ap^r	This study
pME7699	pME6182 with a 3.5-kb fragment containing *rsmY-lacZ*; Gm^r Ap^r	This study
pME7704	pBluescript-II KS containing a *Bam*HI-*Eco*RI 620-bp *retS* upstream region and a *Eco*RI-*Hin*dIII 640-bp *retS* downstream region; Ap^r	This study
pME7705	*Bam*HI-*Hin*dIII fragment (1.2 kb) of pME7704 cloned into *Bam*HI-*Hin*dIII digested pME3087	This study
pME7708	pBluescript-II KS containing a *Bam*HI-*Eco*RI 575-bp *ladS* upstream region and a *Eco*RI-*Hin*dIII 625-bp *ladS* downstream region; Ap^r	This study
pME7709	*Bam*HI-*Hin*dIII fragment (1.2 kb) of pME7708 cloned into *Bam*HI-*Hin*dIII digested pME3087	This study
pUC18	Cloning vector; Ap^r	Vieira and Messing (1991)
pUX-BF13	Helper plasmid containing Tn*7* transposition functions, R6K replicon; Ap^r	Bao *et al.* (1991)
Oligonucleotides	(5'→3')	
RetF1	TATGGATCCGGCCGAGGAAGGCAACGTCTA, with a *Bam*HI underlined site, located 620 bp upstream of the *retS* start codon	
RetF2	TTATGAATTCCGAAATCCCTTCGTTGGTTGA, with an *Eco*RI underlined site, located in the start codon region of *retS*	
RetR1	TTATGAATTCCAGTTGAGCCGACAGGCTCTG, with an *Eco*RI underlined site, located in the stop codon region of *retS*	
RetR2	TATAAAGCTTGACCCCGGTGAAGATGATCTG, with a *Hin*dIII underlined site, located 640 bp downstream of the *retS* stop codon	
RetN1	ACCGCCTGGCGGCCGAGATC, located 190 bp upstream of *retS*	
RetN2	GGCCCACGGTCAGGGGAATG, located 390 bp downstream of *retS*	
LadF1	ATATGGATCCGCATGGCGTAGCCGACTTCAT, with a *Bam*HI underlined site, located 675 bp upstream of the *ladS* start codon	
LadF2	TTATGAATTCCATAGCCGCTGATGGCCATTG, with an *Eco*RI underlined site, located 100 bp upstream the *ladS* start codon	
LadR1	TTATGAATTCGGTCTTGGCACACCCTGAAG, with an *Eco*RI underlined site, located 35 bp downstream of the *ladS* stop codon	
LadR2	TATAAAGCTTGGTGGTGGCACGCACATC, with a *Hin*dIII underlined site, located 660 bp downstream of the *ladS* stop codon	
LadN1	GCGATCTGGCGCCTGCCAGC, located 230 bp upstream of *ladS*	
LadN2	GGCGTCGACCCGCGACTCGG, located 200 bp downstream of *ladS*	

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; MCS, multiple cloning site; Tc^r, tetracycline resistance.

of *P. fluorescens* (Paulsen *et al.*, 2005; Loper *et al.*, 2007). To generate the *retS* mutant CHA1202, a 2.8-kb fragment was deleted in-frame in the chromosomal *retS* gene. For this purpose, a 620-bp *Bam*HI-*Eco*RI fragment upstream of *retS* was amplified by PCR from strain CHA0, using primers RetF1 and RetF2. A 640-bp *Eco*RI-*Hin*dIII fragment including the last 9 bp of *retS* and the adjacent downstream region was amplified by PCR with primers RetR1 and RetR2. The resulting upstream and downstream fragments were cloned by a triple ligation into pBluescript II KS digested with *Bam*HI and *Hin*dIII, giving plasmid pME7704 (Table 1). The 1.2-kb *Bam*HI-*Hin*dIII insert was excised and cloned into the suicide plasmid pME3087 digested with *Bam*HI and *Hin*dIII, producing pME7705 (Table 1). This plasmid was integrated into the chromosome of strain CHA0 by triparental mating, using *E. coli* HB101/pME497 as the mobilizing strain. Clones, in which excision of the vector by a second crossing-over event had occurred, were isolated after enrichment for tetracycline-sensitive cells. The Δ*retS* mutation in the recombinant strain was verified by PCR using primers RetN1 and RetN2.

An analogous gene replacement strategy was followed to create a 2.5-kb *ladS* deletion in strain CHA1204. Using CHA0 DNA as a template, fragments flanking the *ladS* gene were amplified by PCR with primer pairs LadF1-LadF2 and LadR1-LadR2, respectively. The 575-bp upstream and 625-bp downstream fragments obtained were digested with *Bam*HI-*Eco*RI and with *Eco*RI-*Hin*dIII, respectively, and cloned into *Bam*HI-*Hin*dIII-digested pBluescript II KS, resulting in plasmid pME7708 (Table 1). The 1.2-kb *Bam*HI-*Hin*dIII insert was excised from pME7708 and cloned into pME3087 digested with the same restriction enzymes, giving plasmid pME7709 (Table 1), which served to delete the *ladS* gene in strain CHA1204. The Δ*ladS* mutation in the recombinant strain was verified by PCR using primers LadN1 and LadN2.

The Δ*retS* Δ*ladS* double mutant CHA1305 was obtained by using the suicide plasmid pME7705 to delete the *retS* gene in the *ladS* mutant CHA1204. The Δ*retS* Δ*gacS* double mutant CHA1301 (Table 1) was obtained similarly in the *gacS* mutant CHA19. The Δ*ladS* Δ*gacS* double mutant CHA1302 (Table 1) was obtained by using the suicide plasmid pME7709 to delete the *ladS* gene in the *gacS* mutant CHA19. Likewise, pME7705 and pME7709 were used to delete *retS* and *ladS* in the *gacA*::Km[r] mutant CHA89, generating the *gacA*::Km[r] Δ*retS* and the *gacA*::Km[r] Δ*ladS* double mutants CHA1303 and CHA1304, respectively (Table 1). The *retS* and *ladS* mutations were verified by PCR.

The mini-Tn*7*-Gm carrier plasmid pME6182 is a derivative of the previously described Tn*7*-delivery vector pME3280a (Zuber *et al.*, 2003), which carries transcription stop signals at both ends of its multiple cloning site. It was generated in several steps. First, a 0.36-kb *Bfr*I-*Sal*I fragment carrying the transcrip-

tion stop signal located between the *P. aeruginosa pchDCBA* operon and the downstream *ssb* gene (http//www.pseudomonas.com) was cloned between the *Pst*I and *Sal*I sites of pUC18; the *Bfr*I and *Pst*I ends were made compatible by T4 DNA polymerase treatment. The transcription stop signal was then excised as 0.36-kb *Sph*I-*Xba*I fragment and inserted between the *Sph*I and *Spe*I sites of the pME3280a polylinker. To generate the chromosomal insertion of *rsmX-lacZ*, a 3.5-kb *rsmX-lacZ* fusion fragment was first recovered by digesting pME7317 with *Eco*RI and *Xho*I, blunted (with 5 U T4-DNA polymerase (Promega), 100 μM dNTPs, 20 min at room temperature), and cloned into plasmid pME6182 digested with *Sma*I. The resulting plasmid pME7698 and the Tn*7* transposition helper plasmid pUX-BF13 were then coelectroporated into different recipient strains, with selection for the mini-Tn*7*. Likewise, the chromosomal insertion of *rsmY-lacZ* was constructed by digesting pME6916 with *Eco*RI and *Xho*I to recover a 3.5-kb *rsmY-lacZ* fusion fragment, which was blunted and cloned into the plasmid pME6182 digested with *Sma*I. The construct obtained, pME7699, and pUX-BF13 were coelectroporated into different recipient strains.

Antibiotic and HCN assays
Antibiotic production by strain CHA0 was assessed with *Bacillus subtilis* 168M as the indicator as described by Dubuis and Haas (2007) on plates containing, per liter, 10 g proteose peptone, 4.6 g glycerol, 0.75 g K_2HPO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 27 mg $FeCl_3 \cdot 6H_2O$ and 20 g agar (pH 7.0). HCN concentrations in culture supernatants were determined after oxygen-limited growth in NYB according to a protocol previously described (Kay *et al.*, 2006).

β-Galactosidase assays
P. fluorescens strains containing *lacZ* constructs were grown in 20 ml NYB (amended with 0.05% (vol/vol) Triton X-100) in 100-ml Erlenmeyer flasks with shaking. β-Galactosidase activities were quantified by the method of Miller (1972), using cells permeabilized with 5% (vol/vol) toluene. All experiments were performed in triplicate.

Results

Temperature sensitivity of the Gac/Rsm pathway
In rich liquid medium (NYB) and during exponential growth, strain CHA0 had a similar doubling time (33 ± 1 min) at 30 °C as well as at 35 °C. No growth occurred above 37 °C. At an incubation temperature of 35 °C, *P. fluorescens* CHA0 produced only a small amount of antibiotic compounds on rich solid medium, as revealed by a small inhibition zone using a *B. subtilis* overlay. By contrast, at the standard incubation temperature of 30 °C, antibiotic production was markedly stronger. We presume that

the antibiotics that are produced under these conditions include DAPG as a major component. We also noted that strain CHA0 produced less HCN at 35 °C than at 30 °C. Antibiotic and HCN data will be shown in the last section of Results. The expression of the *hcnA* and *phlA* genes involved in the biosynthesis of HCN and DAPG, respectively, revealed the extent of temperature sensitivity. This was seen with translational *hcnA'-'lacZ* and *phlA'-'lacZ* fusions (Figures 1a and b). An *rpoS'-'lacZ* fusion was also tested and found to be less active at 35 °C than at 30 °C (Figure 1c). Together, these results suggested that it might be the Gac/Rsm pathway that responds to temperature as an environmental cue. This hypothesis was confirmed by

the observation that the expression of transcriptional *lacZ* fusions to the *rsmZ*, *rsmY* and *rsmX* sRNA genes was lower at 35 °C than that found at standard 30 °C in NYB medium (Figures 2a–c). Note that strain CHA0 did not tolerate fully induced expression of the *rsmY-lacZ* and *rsmX-lacZ* fusions when these were carried by plasmids having about six copies. In our experience, it is difficult to detect specific activities exceeding 40 000 Miller units, which were the activities found at the end of growth in the wild type carrying pME6916 (*rsmY-lacZ*) or pME7317 (*rsmX-lacZ*; data not shown). To overcome this problem, these fusions were introduced into the
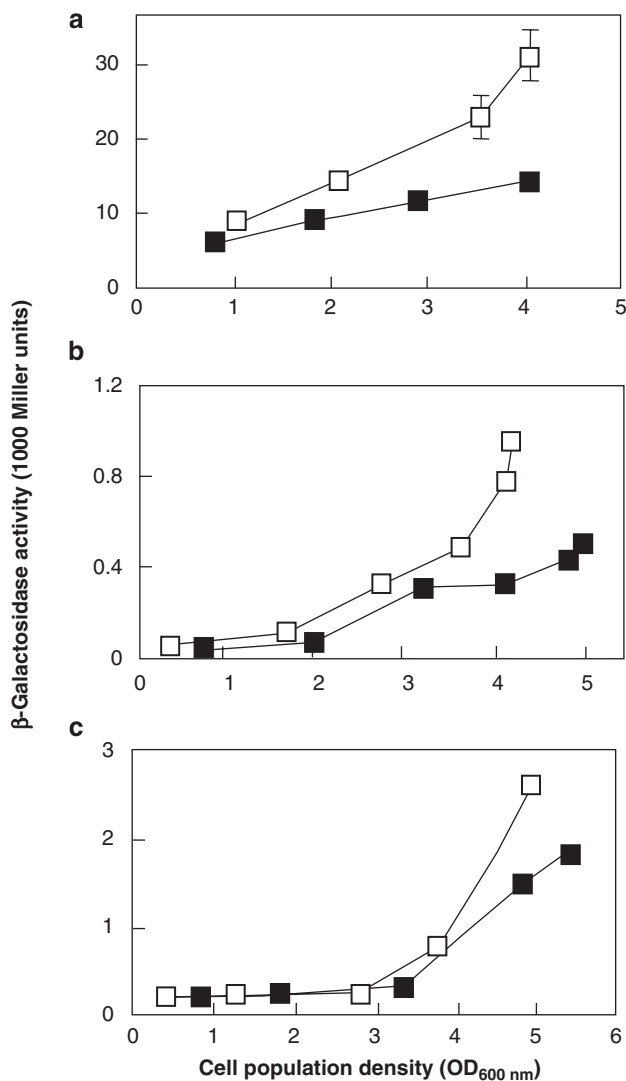

Figure 1 Expression of target genes of the Gac/Rsm cascade in the wild-type CHA0 as a function of growth temperature. Strains were grown in nutrient yeast broth (NYB) at 30 or 35 °C. β-Galactosidase activities were determined for (**a**) a p*tac-hcnA'-'lacZ* fusion (on pME6530), (**b**) a *phlA'-'lacZ* fusion (on pME6259) and (**c**) an *rpoS'-'lacZ* fusion (on pME6355). □, wild type at 30 °C; ■, wild type at 35 °C. Experiments were done three times; each value is the average ± standard deviation.
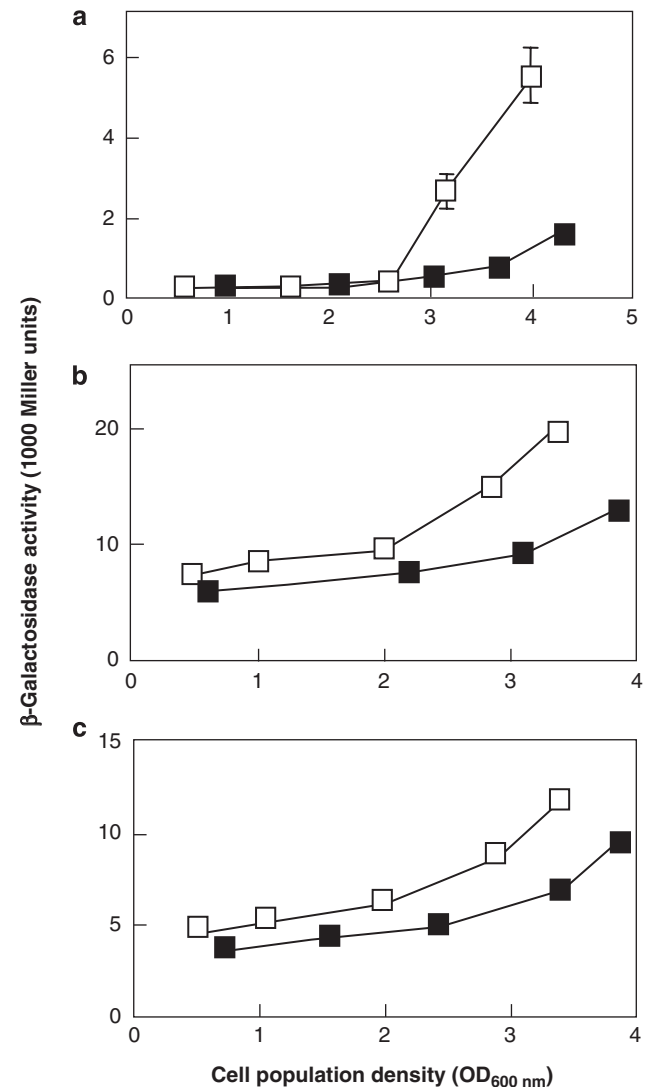

Figure 2 Influence of the growth temperature on transcriptional expression of the small regulatory RNAs RsmZ, RsmY and RsmX in the wild-type CHA0. Strains were grown in nutrient yeast broth (NYB) at 30 or 35 °C. β-Galactosidase activities were determined for (**a**) a plasmid-borne chromosomal *rsmZ-lacZ* fusion (on pME6091), (**b**) a chromosomal *rsmY-lacZ* fusion and (**c**) a chromosomal *rsmX-lacZ* fusion. □, wild type at 30 °C; ■, wild type at 35 °C. Experiments were done three times; each value is the average ± standard deviation.

chromosomal Tn*7* attachment site, whereas *rsmZ-lacZ* was assessed on a plasmid construct.

RetS as a temperature-sensitive element

To test which component of the Gac/Rsm pathway was responsible for the temperature effect, we first tested the *rsmZ-lacZ* fusion in the *gacS*Δ76 mutant CHA19.8, which expresses GacA-dependent genes at constitutive high levels (Zuber *et al.*, 2003). However, the *rsmZ-lacZ* construct displayed no temperature sensitivity (data not shown), indicating that the activities of the GacS sensor and the GacA response regulator were not compromised at high temperature. This led us to inspect the potential influence of the two accessory sensors LadS and RetS, which had previously been described in *P. aeruginosa* (Goodman *et al.*, 2004, 2009; Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). To this end, we constructed mutants deleted for *ladS* (PFL_5426 in the closely related strain Pf-5) or *retS* (PFL_0664 in strain *P. fluorescens* Pf-5) in the wild-type *P. fluorescens* CHA0. In a *ladS* mutant, the *lacZ* fusions to *rsmZ*, *rsmY* and *rsmX* were all expressed at levels (Figures 3a–c) that were roughly half of those found in the wild type (Figures 2a–c). However, in the *ladS* background temperature sensitivity essentially persisted (Figure 3). By contrast, in a *retS* mutant, temperature sensitivity was lost and all three fusion constructs were expressed at high levels, well above those observed in the wild type (Figures 4a–c). In a *retS ladS* double mutant,
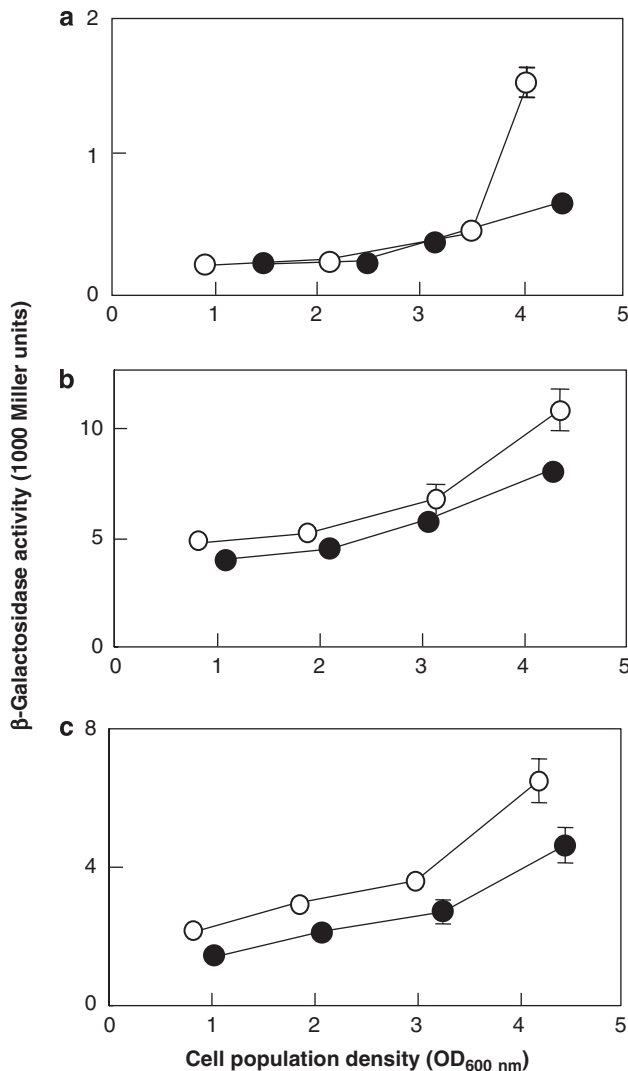

Figure 3 Influence of the growth temperature on transcriptional expression of the small regulatory RNAs RsmZ, RsmY and RsmX in a *ladS* mutant. Strains were grown in nutrient yeast broth (NYB) at 30 or 35 °C. β-Galactosidase activities were determined for (**a**) a plasmid-borne *rsmZ-lacZ* fusion (on pME6091), (**b**) a chromosomal *rsmY-lacZ* fusion and (**c**) a chromosomal *rsmX-lacZ* fusion. ○, *ladS* mutant at 30 °C; ●, *ladS* mutant at 35 °C. Experiments were done three times; each value is the average ± standard deviation.
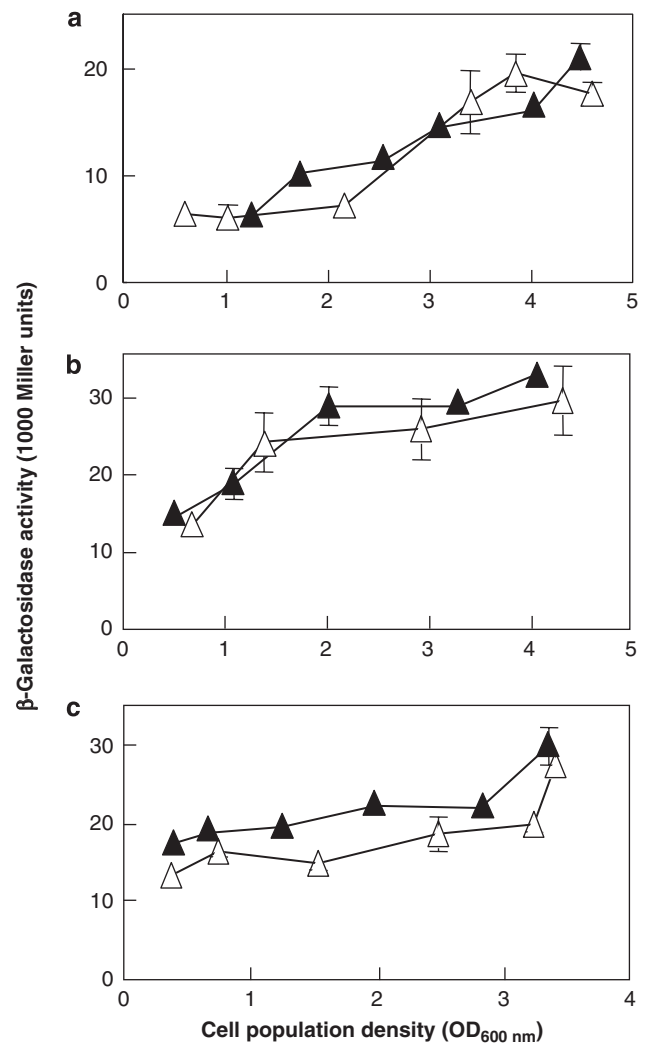

Figure 4 Influence of the growth temperature on transcriptional expression of the small regulatory RNAs RsmZ, RsmY and RsmX in a *retS* mutant. Strains were grown in nutrient yeast broth (NYB) at 30 or 35 °C. β-Galactosidase activities were determined for (**a**) a plasmid-borne *rsmZ-lacZ* fusion (on pME6091), (**b**) a chromosomal *rsmY-lacZ* fusion and (**c**) a chromosomal *rsmX-lacZ* fusion. △, *retS* mutant at 30 °C; ▲, *retS* mutant at 35 °C. Experiments were done three times; each value is the average ± standard deviation.

the same result was obtained as in a *retS* mutant (data not shown). A control experiment using a transcriptional *lacZ* fusion to the constitutive *tac* promoter confirmed that transcription *per se* was not compromised at 35 °C (data not shown). Taken together, these results indicate that LadS and RetS have positive and negative effects, respectively, on the Gac/Rsm cascade of *P. fluorescens* CHA0 and that temperature sensitivity depends essentially on RetS.

Mutations in gacS *or* gacA *are epistatic to mutations in* ladS *and* retS

Recent studies indicate that LadS and RetS physically interact with GacS (Goodman *et al.*, 2009; Workentine *et al.*, 2009). The following experiments lend support to these findings. The expression of *rsmZ*, *rsmY* and *rsmX* was very low in both *gacS* and *gacA* mutants of strain CHA0, compared with the wild type, in agreement with previous results (Kay *et al.*, 2005). In *ladS gacS*, *ladS gacA*, *retS gacS* and *retS gacA* double mutants, the expression of the three sRNA genes was equally low (Supplementary Figure S1). By contrast, as shown above (Figures 2–4), in a simple *ladS* background all reporter fusions were expressed at levels that were intermediate between the basal (*gacS* or *gacA*) level and the wild-type level, whereas very high levels occurred in a simple *retS* mutant. Thus, LadS and RetS functioned as modulators of the Gac/Rsm pathway in *P. fluorescens* CHA0. The fact that *gacS* and *gacA* mutations were epistatic to *retS* and *ladS* mutations are in agreement with a model in which RetS and LadS make contacts with GacS.

A retS *mutant shows diminished temperature regulation of biocontrol factor expression*

If RetS–GacS interaction is regulated by temperature, we would expect that in a *retS* mutant high temperature should have little or no effect on the expression of biocontrol factors. This was the case. The expression of translational *lacZ* fusions to the *hcnA* and *phlA* target genes was consistent with the expression of the *rsmZ*, *rsmY* and *rsmX* sRNA genes: a *ladS* mutation lowered the expression, whereas a *retS* mutation increased the expression of these *lacZ* fusions (Supplementary Figure S2), although the *hcnA'-'lacZ* reporter on plasmid pME6530 could not be measured in the *retS* mutant, because β-galactosidase levels were above the tolerated upper limit. The translational expression of *rpoS* also followed the same pattern (Supplementary Figure S2). Antibiotic production was enhanced in the *retS* mutant CHA1202, relative to that found in the wild type. This effect was most pronounced at 35 °C where the wild type had low antibiotic activity, as revealed by a small zone of inhibition of *B. subtilis* (Figure 5). The effects of the *ladS* mutation on antibiotic production (Figure 5) were also consistent with
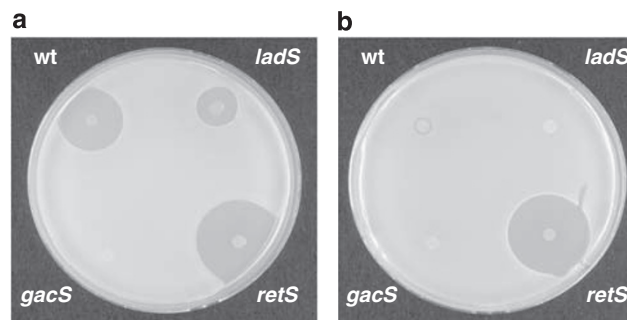
Figure 5 Influence of the growth temperature on the production of antibiotics in the *Pseudomonas fluorescens* wild-type strain CHA0, the *retS* mutant CHA1202, the *ladS* mutant CHA1204 and the *gacS* mutant CHA19. Antibiotic production was assessed by spotting 2-μl samples of an overnight culture (adjusted to $OD_{600} = 2.0$) onto solid plates (see Materials and methods). After incubation at 30 °C (**a**) or 35 °C (**b**) for 12–14 h, *P. fluorescens* was killed by UV irradiation and *Bacillus subtilis* strain 168 M was overlaid in 0.5% nutrient yeast broth (NYB) agar. Plates were incubated at 30 °C until inhibition halos appeared.
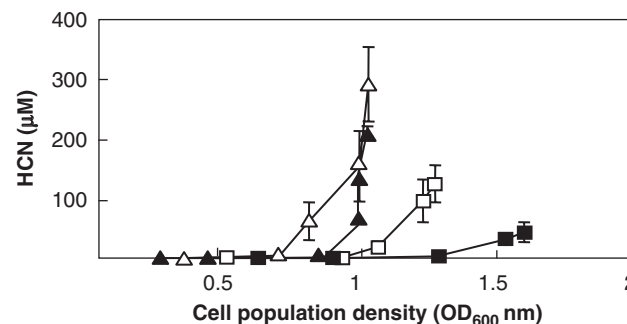
Figure 6 Influence of the growth temperature on hydrogen cyanide (HCN) production in the *Pseudomonas fluorescens* wild-type strain CHA0 and in the *retS* mutant CHA1202. HCN production was measured in nutrient yeast broth (NYB) medium in which *P. fluorescens* had been grown with oxygen limitation. □, wild type at 30 °C; ■, wild type at 35 °C; △, *retS* mutant at 30 °C; ▲, *retS* mutant at 35 °C. Experiments were done five times; each value is the average ± standard deviation.

sRNA expression data (Figure 3). Finally, HCN production was enhanced and advanced in the *retS* mutant both at 30 °C and at 35 °C, whereas in the wild-type HCN formation was delayed and strongly reduced at 35 °C (Figure 6). The model shown in Figure 7 summarizes the findings and will be presented in Discussion.

Discussion

The principal aim of this study was to shed light on the mechanism involved in thermoregulation of biocontrol factor expression in *P. fluorescens* CHA0. In precedent work on various biocontrol strains of *P. fluorescens* phenotypic evidence had been obtained for this type of regulation (Shanahan *et al.*, 1992; Slininger and Shea-Wilbur, 1995; Schmidt *et al.*, 2004), but the mode of action
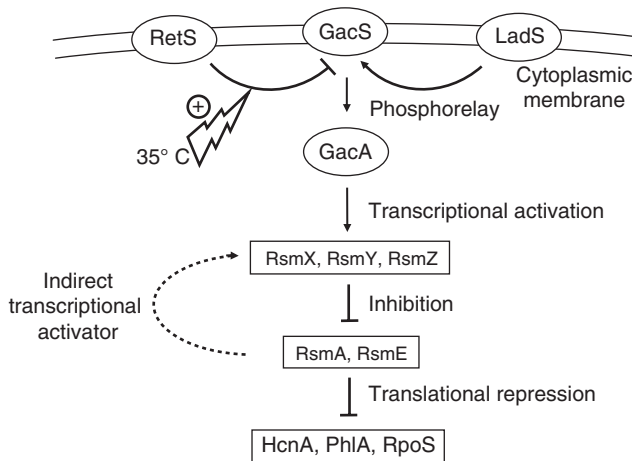
Figure 7 Integrative model of the Gac/Rsm signal transduction pathway in *Pseudomonas fluorescens* CHA0. ↓, positive control; ⊥, negative control; dotted lines, indirect control. Evidence for this pathway comes from this work and from previous studies (Heeb *et al.*, 2002, 2005; Valverde *et al.*, 2003; Kay *et al.*, 2005; Reimmann *et al.*, 2005).

remained unknown. Our observation that key regulatory elements, the sRNA genes *rsmX*, *rsmY* and *rsmZ*, all showed diminished transcription at 35 °C, relative to that seen at the standard growth temperature of 30 °C (Figure 2), led to the hypothesis that some component of the Gac/Rsm signal transduction pathway might be temperature-sensitive. We found that a *retS* mutant had lost temperature sensitivity and expressed the sRNA genes at constitutive high levels (Figure 4). This finding suggests that RetS activity is influenced by temperature and that the GacS/GacA two-component system itself remains fully functional at elevated temperature. The ecological significance of temperature-sensitive expression of biocontrol factors is speculative at the moment. If we admit that the production of biocontrol traits normally confers a selective advantage on the producer strains (Haas and Keel, 2003), then we can assume that this advantage may not be relevant at temperatures around 35 °C where the growth of many competing soil microorganisms including some pathogenic fungi is inhibited. Under these conditions, *P. fluorescens* can conceivably afford to dedicate its metabolic energy essentially to primary metabolism, without having to pay the full cost of secondary metabolism, which is necessary for producing biocontrol factors.

We also tested pH as a potential cue, as a previous study (Mondragón *et al.*, 2006) had shown pH-sensitivity of the BarA/UvrY two-component system in *E. coli*. However, at pH 6.2, the lowest pH value allowing good growth of strain CHA0, the expression of *lacZ* fusions to *rsmZ*, *rsmY* and *rsmX* was similar to that measured at standard neutral pH (data not shown).

The specificity of the GacS–GacA interaction has previously been substantiated by genetic analyses conducted in several *Pseudomonas* species (Rich

et al., 1994; Heeb and Haas, 2001) and by biochemical evidence for phosphotransfer between a soluble form of GacS and GacA in *P. aeruginosa* (Goodman *et al.*, 2009). In *P. aeruginosa*, the LadS and RetS sensors have been reported to modify the activity of the GacS/GacA system *in vivo* (Goodman *et al.*, 2004; Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). LadS contains eight putative transmembrane segments, an autophosphorylation (kinase) domain and a response regulator domain. Mutation of the *ladS* gene strongly diminished *rsmZ* expression (*rsmY* expression was not tested) and resulted in reduced production of adhesive extracellular polysaccharides and enhanced expression of the type III secretion apparatus in *P. aeruginosa* (Ventre *et al.*, 2006). Thus, LadS appears to activate the GacS/GacA system although the mechanism by which this effect is brought about has not been elucidated. In *P. fluorescens* CHA0, there is evidence that LadS and GacS may interact physically (Workentine *et al.*, 2009). As we have shown here, LadS positively controls *rsmX*, *rsmY* and *rsmZ* expression, albeit less strongly than GacS (Figures 2, 3; Supplementary Figure S2). As both *gacS* and *gacA* mutations were epistatic to a *ladS* mutation (Supplementary Figure S1), it is likely that LadS acts upstream of GacS. This might be achieved if LadS physically interacted with GacS and thereby facilitated autophosphorylation of GacS (see model in Figure 7). A direct interaction between LadS and GacA appears less plausible as in this case the expression of the three sRNAs should be more strongly affected by the double *ladS gacS* mutation than by single *ladS* or *gacS* mutations; however, this was not observed (Supplementary Figure S1).

The RetS sensor of *P. aeruginosa* also contains eight putative transmembrane segments, a kinase domain and an adjacent response regulator domain. However, RetS has an additional response regulator domain located at the C terminus, and this domain is most important for biological activity. A *retS* mutant of *P. aeruginosa* overexpressed *rsmZ* and biofilm exopolysaccharides and had a downregulated type III secretion apparatus; *rsmY* expression was not tested (Goodman *et al.*, 2004; Laskowski and Kazmierczak, 2006). Mutation in *gacS* was found to be epistatic to mutation in *retS* (Goodman *et al.*, 2004). RetS acts as an antagonist of GacS *in vitro* (Goodman *et al.*, 2009). In *P. fluorescens* CHA0, we found that the RetS homologue had a negative effect on the expression of all three GacA-controlled sRNA genes, *rsmX*, *rsmY* and *rsmZ* (Figures 2 and 4). Consequently, a *retS* mutation resulted in strongly enhanced promoter activities of the three sRNA genes and hence the expression of target genes and of biocontrol factors was strongly elevated as well (Figures 5, 6; Supplementary Figure S2). These data are consistent with a model of direct RetS–GacS interaction (Figure 7), for which there is *in vivo* evidence in strain CHA0 (Workentine *et al.*,

2009). However, the biochemistry of the antagonistic interaction between RetS and GacS is still uncertain. It has been postulated that RetS could have phosphatase activity on GacS (Laskowski and Kazmierczak, 2006), but this has not been confirmed *in vitro* (Goodman *et al.*, 2009). There are precedents of interacting membrane sensor proteins: various dimeric chemoreceptor proteins of enteric bacteria are known to assemble as trimers in the cytoplasmic membrane, allowing the bacteria to integrate several signals in the chemotactic response (Hazelbauer *et al.*, 2008). Note that the model shown in Figure 7 differs from another recently published model in which GacS, LadS and RetS are all assumed to interact with GacA in *P. aeruginosa* (Gooderham and Hancock, 2009).

Temperature is an important environmental cue. Some bacteria can sense it via transmembrane sensor kinases other than RetS. For example, a temperature-responsive sensor regulates the production of the chlorosis-inducing toxin coronatine in the soybean pathogen *Pseudomonas syringae* pv. glycinea PG4180. Toxin production occurs at 18 °C but not at 28 °C, the optimal growth temperature (Palmer and Bender, 1993). This thermoregulation is mediated at the transcriptional level by a regulatory system consisting of a histidine protein kinase, CorS, and two transcriptional activators, CorR and CorP. The C-terminal cytosolic region of CorS appears to act as a temperature sensor; it is believed to respond to intracellular temperature changes via autophosphorylation and to transduce the signal to the response regulator CorR via phosphorylation (Braun *et al.*, 2008). Another example is provided by the thermal control of fatty acid synthesis. The fraction of unsaturated phospholipid acyl chains in phospholipids increases when the growth temperature decreases. This adaptation improves membrane fluidity. In *B. subtilis*, a two-component system composed of a membrane-associated kinase, DesK, and a soluble transcriptional activator, DesR, regulates the transcription of the *des* gene coding for a Δ^5-fatty acid desaturase. DesK is a sensor having both kinase and phosphatase activities. At 37 °C, when membrane lipids are in a disordered fluid state, the phosphatase mode of DesK is dominant. After a temperature downshift to 25 °C, the proportion of ordered membrane lipids (that is, a nonfluid state) predominates, leading to an increase of the kinase mode of DesK. This results in autophosphorylation and transfer of the phosphoryl group to DesR. Phosphorylated DesR activates transcription of *des*, and the Des enzyme introduces a double bond into the acyl chains of membrane phospholipids (Aguilar *et al.*, 2001). Experimental evidence points to membrane fluidity being a stimulus of the N-terminal transmembrane domain of DesK (Hunger *et al.*, 2004).

In this study, we have shown that RetS is a stronger antagonist of GacS at 35 °C than at 30 °C. This mechanism of temperature sensing might involve a change in membrane fluidity, enabling a stronger contact between the two sensors at 35 °C. Previous observations in *Pseudomonas* species have indeed suggested that changes of membrane fluidity can modify quorum sensing regulation (Baysse and O'Gara, 2007).

Acknowledgements

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