ELSEVIER

FEMS Microbiology Letters 243 (2005) 251–258

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0

Stephan Heeb [a,b,*], Claudio Valverde [a,c], Cécile Gigot-Bonnefoy [a], Dieter Haas [a]

[a] *Département de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland*
[b] *Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, UK*
[c] *Departamento de Ciencia y Tecnología, Programa de Investigación sobre Interacciones Biológicas, Universidad Nacional de Quilmes, Saenz Peña 180 Bernal B1876BXD, Argentina*

Received 22 July 2004; received in revised form 8 December 2004; accepted 9 December 2004

First published online 16 December 2004

Edited by S. Silver

Abstract

In *Pseudomonas fluorescens* biocontrol strain CHA0, the two-component system GacS/GacA positively controls the synthesis of extracellular products such as hydrogen cyanide, protease, and 2,4-diacetylphloroglucinol, by upregulating the transcription of small regulatory RNAs which relieve RsmA-mediated translational repression of target genes. The expression of the stress sigma factor σ^S (RpoS) was controlled positively by GacA and negatively by RsmA. By comparison with the wild-type CHA0, both a *gacS* and an *rpoS* null mutant were more sensitive to H_2O_2 in stationary phase. Overexpression of *rpoS* or of *rsmZ*, encoding a small RNA antagonistic to RsmA, restored peroxide resistance to a *gacS* mutant. By contrast, the *rpoS* mutant showed a slight increase in the expression of the *hcnA* (HCN synthase subunit) gene and of the *aprA* (major exoprotease) gene, whereas overexpression of σ^S strongly reduced the expression of these genes. These results suggest that in strain CHA0, regulation of exoproduct synthesis does not involve σ^S as an intermediate in the Gac/Rsm signal transduction pathway whereas σ^S participates in Gac/Rsm-mediated resistance to oxidative stress.

Keywords: *Pseudomonas fluorescens*; RpoS; GacA; RsmA; Secondary metabolism; Oxidative stress

1. Introduction

The regulation and functions of the stress and stationary phase sigma factor σ^S (also known as σ^{38}) encoded by *rpoS*, have been studied in a variety of Gram-negative bacteria, especially in *Escherichia coli* [1,2] and *Pseudomonas* spp. [3–7]. In *E. coli*, σ^S induces more than 40 genes or operons during the transition from exponential to stationary phase, producing proteins usually associated with resistance to stress [1,2]. The regulation of *rpoS* is very complex and involves transcriptional, translational and post-translational control mechanisms. At the transcriptional level, *rpoS* expression appears to be modulated by the sensor kinase BarA (a GacS homologue) [8], intracellular levels of cAMP and its receptor protein CRP, guanosine-3′,5′-bispyrophosphate (ppGpp), inorganic polyphosphate, and UDP–glucose [9–11]. Translational *rpoS* control responds to

* Corresponding author. Tel.: +44 115 951 50 89; fax: +44 115 846 79 51.
E-mail address: stephan.heeb@nottingham.ac.uk (S. Heeb).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.12.008

changes in osmolarity, temperature, and nutrient deprivation, e.g. during transition to stationary phase, and is exerted on the 5′ untranslated leader of *rpoS* mRNA [2]. Translation of *rpoS* is modulated by the RNA-binding protein Hfq [12], the histone-like protein H-NS [13], and the small regulatory RNAs DsrA, OxyS, and RprA [14–16]. Post-translational control involves σ^S turnover and is mediated essentially by the intracellular ClpXP protease [2], the heat shock chaperone DnaK [17], and the response regulator RssB [18]. Additionally, the activity of σ^S is subject to competition with other sigma factors for association with core RNA polymerase to form the RNA polymerase holoenzyme [19].

Certain functions of RpoS are similar in *Pseudomonas* spp. and in enteric bacteria. In particular, survival during osmotic, heat or oxidative stresses is reduced in *rpoS* mutants of *P. aeruginosa*, *P. putida*, and *P. fluorescens* [4–7,20]. However, in *Pseudomonas* spp., *rpoS* appears to be more extensively regulated at the transcriptional level than in *E. coli*. The TetR-like regulator PsrA, which strongly activates *rpoS* transcription by binding to a palindromic sequence in the *rpoS* promoter, has been found so far only in *Pseudomonas* spp. [21–23]. In *P. fluorescens* Pf-5 and *P. chlororaphis* O6, but not in *P. aeruginosa*, *rpoS* expression is positively controlled by the GacA/GacS two-component system and an *rpoS* null mutation negatively affects the survival under conditions of oxidative stress [4,22,24–26].

In the biocontrol strain *P. fluorescens* CHA0 [27], the *rpoS* gene is located immediately upstream of *rsmZ*, a GacA/GacS-controlled gene encoding a small regulatory RNA that antagonizes translational repression of target genes by the RNA-binding protein RsmA [28]. Typical Gac/Rsm-controlled target genes encode exoproducts involved in biocontrol, e.g. *hcnA* (for HCN synthesis), *phlA* (for 2,4-diacetylphloroglucinol [Phl] synthesis) and *aprA* (for the major exoprotease) [29–31]. In *Erwinia carotovora*, RpoS negatively affects the production of extracellular enzymes, apparently by upregulating *rsmA* expression [32]. In *Azotobacter vinelandii*, GacA and RpoS are part of a cascade controlling alginate production [33]. These observations prompted us to investigate the question whether RpoS could be an intermediate in the Gac/Rsm signal transduction pathway of *P. fluorescens* CHA0, in the regulation of exoproduct formation and in resistance to oxidative stress.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. fluorescens CHA0 derivatives [27] and *E. coli* DH5α [34] were routinely grown in nutrient yeast broth (NYB; 2.5% [w/v] nutrient broth, 0.5% [w/v] yeast extract) with shaking, or on nutrient agar [28] amended with the following antibiotics, when required: ampicillin, 100 μg ml^{-1} (for *E. coli*); tetracycline, 25 μg ml^{-1} (125 μg ml^{-1} for *P. fluorescens*); and gentamicin, 10 μg ml^{-1}. Routine incubation temperatures were 37 ° C for *E. coli* and 30 °C for *P. fluorescens*. When *P. fluorescens* was to receive heterologous DNA (e.g., in electroporation [28,35]), the incubation temperature was raised to 35 °C. Expression of a *phlA′–′lacZ* fusion in *P. fluorescens* was monitored in minimal medium OS amended with glucose and ammonium [35].

2.2. DNA manipulations

Small-scale plasmid extractions were performed by the cetyltrimethylammonium bromide method [36], and large-scale preparations were done by using the Qiagen Plasmid Midi kit (Qiagen Inc.). DNA fragments were purified from agarose gels with the Geneclean II Kit (Bio101, La Jolla, CA). DNA sequencing was performed with the Big Dye Terminator Cycle sequencing kit and an ABI-Prism 373 automatic sequencer (Applied Biosystems). PCRs were typically carried out with 2.5 U of thermostable DNA polymerase (Extra-Pol II; Eurobio) in a reaction mixture containing 100 ng of target DNA, 250 μM of each of the four dNTPs (Roche), 10 pmol of each primer, 5 mM $MgCl_2$, and 1× Extra-Pol buffer in a final volume of 20 μl. For the amplification reaction, 25 cycles (1 min at 94 °C, 1 min at 50–55 °C (depending on the G + C content of the primers), and 1 min at 72 °C) were followed by a final elongation step of 5 min at 72 °C.

2.3. Overexpression of rpoS

The 2.6-kb *Hin*dIII-*Eco*RI *rpoS rsmZ* fragment from pME6087 [28] was inserted into pBluescript II KS+ [Stratagene] to produce pME6088 (Fig. 1), and a PCR with oligonucleotides RPOSCHA04 and RPOSCHA05 was done to amplify a 1.1-kb *Eco*RI–*Bgl*II *rpoS* fragment from this plasmid. RPOSCHA04 (5′-CCGGAATTCGAACTCACCAAAGGACTATAAC-3′) anneals upstream of the *rpoS* gene and creates an artificial *Eco*RI site (underlined) 28 nucleotides upstream of the start codon. RPOSCHA05 (5′-CGAGTAGATCTGGGCTCTTGTGAATCGATC-3′) anneals downstream of the coding region and creates an artificial *Bgl*II site (underlined) 26 nucleotides behind the stop codon. The *Eco*RI–*Bgl*II fragment was inserted into *Eco*RI–*Bam*HI-digested pME6001 [37] to produce pME6354, placing *rpoS* under control of the *lac* promoter (Fig. 1).

2.4. Construction of an rpoS′–′lacZ translational fusion

PCR with oligonucleotides RPOSCHA03 and T7 [28] was done to amplify a 0.96-kb *Eco*RI–*Xho*I *rpoS′* fragment from pME6088. RPOSCHA03 anneals to the 5′

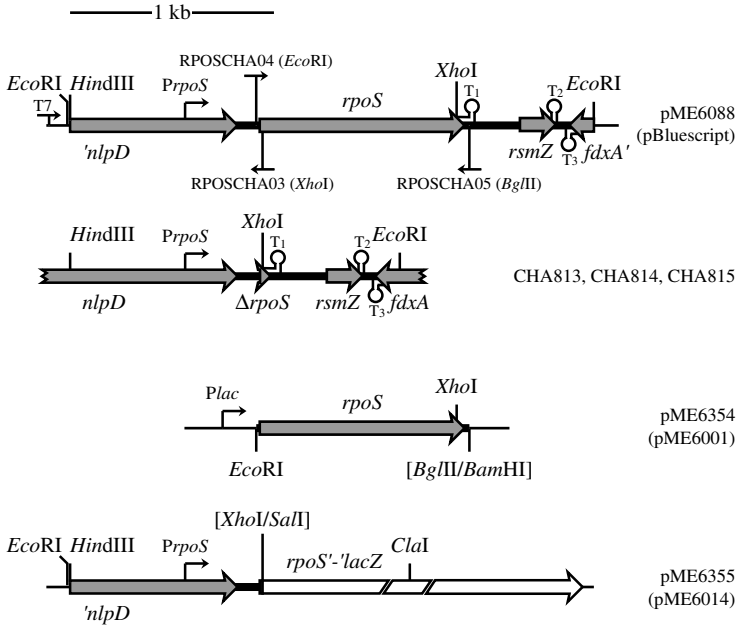

Fig. 1. Maps of constructs involving *rpoS* from *P. fluorescens* CHA0 used in this study. Vectors used for the construction of pME6088, pME6354, and pME6355 are shown in brackets. In strains CHA813 (chromosomal *aprA'–'lacZ* fusion), CHA814 (chromosomal *hcnA'–'lacZ*) and CHA815 (wild-type background) *rpoS* codons 8–322 (out of 336) were deleted in-frame [28]. In pME6354, *rpoS* was placed under the *lac* promoter (P_{lac}) of vector pME6001. Plasmid pME6355 carries a translational *rpoS'–'lacZ* fusion at the 8th codon. P_{rpoS}: *rpoS* promoter; T1, T2 and T3: *rho*-independent transcriptional terminators. Restriction sites that were used for the constructions are shown together with the positions and orientations (5′ → 3′) of oligonucleotides used to amplify fragments. Nucleotide sequence of the 2.6-kb *Hin*dIII–*Eco*RI fragment has been deposited in GenBank under Accession No. AF245440.

end of *rpoS* and creates an artificial *Xho*I site at the 8th codon [28]. The PCR product was digested with *Eco*RI and *Xho*I and inserted into pBluescript II KS+ to produce pME6350, where it was sequenced to confirm the absence of mutations. This insert was then subcloned into *Eco*RI–*Sal*I-cut pNM482 [38], and the resulting 1.8-kb *Eco*RI–*Cla*I *rpoS'–'lacZ'* fragment was finally inserted into *Eco*RI–*Cla*I-cut pME6014 [35] to produce pME6355 (Fig. 1).

2.5. β-Galactosidase assays

β-Galactosidase activities in *P. fluorescens* carrying *lacZ* fusion constructs were quantified by the Miller method [39], using cells permeabilised with 5% [v/v] toluene. All measurements were done after inoculation of 30 ml of NYB with 300 μl of overnight cultures and incubation at 30 °C with shaking.

2.6. Detection of RsmA by Western blotting

Strains were grown in 20 ml of NYB containing 0.05% [v/v] Triton X-100 in 125-ml Erlenmeyer flasks at 30 °C with agitation. An equivalent of 0.4 OD_{600} units from cultures in stationary phase cells was centrifuged. Cells were washed with 0.9% NaCl [w/v], resuspended in 20 μμl of loading buffer (50 mM Tris–HCl pH 6.8, 2% [w/v] SDS, 0.1% [w/v] bromophenol blue, 15% [w/v] glycerol, 5% [v/v] 2-mercaptoethanol) and immediately treated at 100 °C for 10 min. For each sample, 15 μl of cell lysate was loaded per well. Samples were fractionated by Tricine–SDS–PAGE in 16% [w/v] acrylamide:bisacrylamide 29:1 gels at 75 V during 4 h [40]. After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore) at 50 mA and 4 °C for 1 h. RsmA was detected using polyclonal antibodies raised against purified *Yersinia enterocolitica* RsmA as previously described [41]. Membranes were developed with the ECL Western Blotting analysis system (Amersham-Pharmacia) following the manufacturer's instructions.

2.7. Assay of oxidative stress survival

Stationary phase cells of *P. fluorescens* grown in NYB were washed and resuspended in 0.9% [w/v] NaCl to an OD_{600} of 0.8. This suspension (5 ml) was exposed to 40 mM H_2O_2 at 30 °C with agitation, and the number of culturable cells per ml was determined on nutrient agar before and after 1 h of exposure.

3. Results

3.1. Expression of rpoS is regulated by GacA and RsmA

In *P. fluorescens* CHA0, like in most pseudomonads, the *rpoS* gene is flanked by the *nlpD* (for a lipoprotein)

and *rsmZ* genes [28] (Fig. 1). The position of the *rpoS* promoter, located in the *nlpD* coding region, was deduced by sequence comparison with the highly conserved *rpoS* promoters previously described in *P. aeruginosa* [42] and *P. putida* WCS358 [21]. A fragment containing the *rpoS* promoter and the first 8 codons of *rpoS* was joined to a *'lacZ* fragment in pME6355, creating an *rpoS'–'lacZ* translational fusion (Fig. 1). In the wild-type CHA0, the expression of the *rpoS'–'lacZ* fusion was induced at high cell population densities and was about 4-fold higher than in the *gacA* mutant CHA89 [43] (Fig. 2(a)). A similar positive effect of the GacS/GacA system has been observed with a chromosomal *rpoS lacZ* transcriptional fusion in *P. fluorescens* strain Pf-5 [24]. In the *rsmA* mutant CHA809 [28], β-galactosidase activities were elevated about 2-fold and occurred at lower cell population densities compared with the wild-type CHA0 (Fig. 2(a)), suggesting that RsmA acts as a negative control element in the expression of *rpoS*.

3.2. *Effects of deletion and overexpression of* rpoS *on* hcnA*,* aprA*, and* phlA *expression*

In strain CHA0, the expression of typical exoproduct genes such as *hcnA*, *aprA*, and *phlA* is 30- to 60-fold higher than in *gacS* or *gacA* mutants [28,31,35,37]. If these positive effects of the GacS/GacA system were mediated, at least in part, by RpoS, we would expect to see reduced expression of *hcnA*, *aprA*, and *phlA* in an *rpoS*-negative background. However, in the *rpoS* deletion strains CHA814 [28] (*hcnA'–'lacZ*Δ*rpoS*) and CHA813 [28] (*aprA'–'lacZ* Δ*rpoS*) a slight (⩽30%) increase in β-galactosidase activities was found with both reporter fusions when compared with the *rpoS*$^+$ strains CHA207 [37] (*hcnA'–'lacZ*) and CHA805 [37] (*aprA'–'lacZ*) (Fig. 2(b)). This small effect appeared late in growth (Fig. 2(b)). The growth rates were unaltered by the *rpoS* mutation under the conditions used. The result obtained with the*hcnA'–'lacZ* fusion is in harmony with the observation that an *rpoS* mutant of *P. fluorescens* Pf-

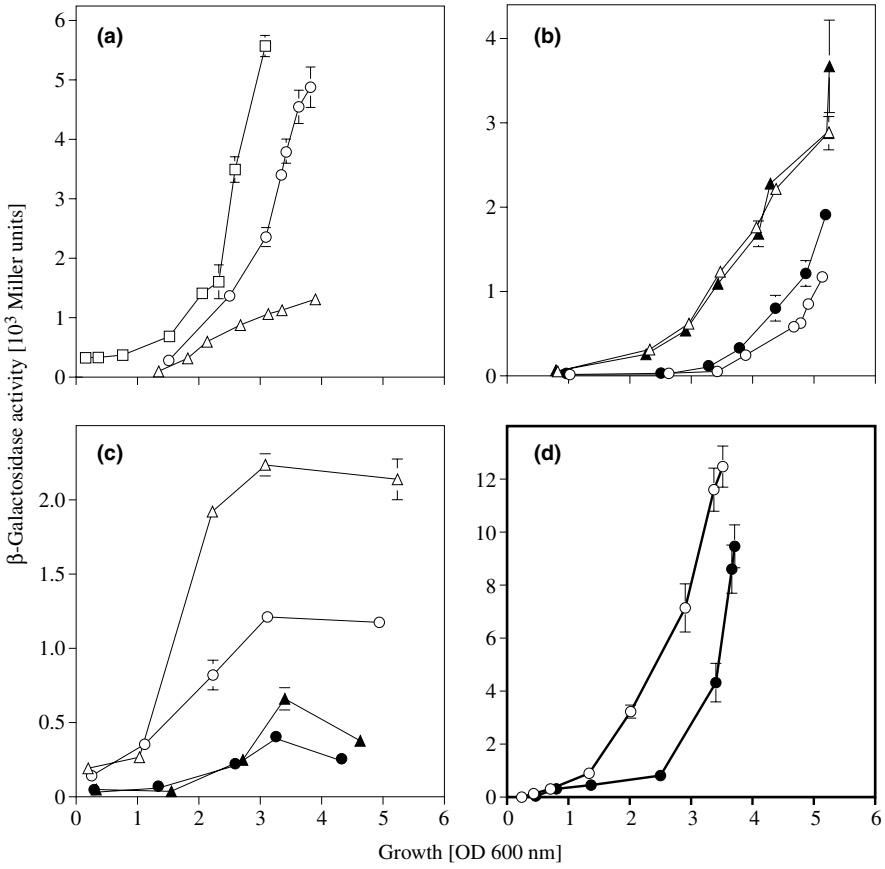

Fig. 2. Regulation of *rpoS*, *hcnA*, *aprA*, and *phlA* in the Gac/Rsm system. (a) β-Galactosidase activities specified by pME6355 (translational *rpoS'–'lacZ* fusion) were assayed in the *P. fluorescens* wild-type CHA0 (○), the *gacA* mutant CHA89 (△) and the *rsmA* mutant CHA809 (□). (b) β-Galactosidase activities were determined in the RpoS-negative strains CHA814 (*hcnA'–'lacZ* Δ*rpoS*; ▲) and CHA813 (*aprA'–'lacZ* Δ*rpoS*; ●) as well as in the *rpoS*$^+$ strains CHA207 (*hcnA'–'lacZ*; △) and CHA805 (*aprA'–'lacZ*; ○). (c) β-Galactosidase expression of chromosomal translational *aprA'–'lacZ* (●,○) or *hcnA'–'lacZ* (▲,△) fusions were tested in a wild-type background carrying the control vector pME6001 (○,△) or the *rpoS*-overexpressing plasmid pME6354 (●,▲). (d) β-Galactosidase activity produced by a translational *phlA'–'lacZ* fusion on pME6259 was tested in the *P. fluorescens* wild-type strain CHA0 (●) and the *rsmA* mutant CHA809 (○). Activities (Miller units) are mean values of triplicate experiments ± SD.

5 overproduces the extracellular metabolite HCN [24]. To monitor *phlA* expression, the *phlA′–′lacZ* translational fusion carried by pME6259 [35] was introduced into the wild type and the *rpoS* mutant CHA815 [28]. No significant difference was found with this fusion between the two backgrounds (data not shown).

When plasmid pME6354, in which *rpoS* is under the control of the *lac* promoter (Fig. 1), was introduced into strains CHA207 and CHA805, β-galactosidase activities produced by the *hcnA′–′lacZ* and *aprA′–′lacZ* translational fusions were reduced ⩾3-fold during growth, compared to the control strains containing the vector pME6001 (Fig. 2(c)), suggesting a negative control of HCN and protease production by RpoS.

3.3. Negative control of phlA expression by RsmA

In our previous studies we have shown that RsmA negatively controls the expression of the *hcnA* and *aprA* genes at a post-transcriptional level in strain CHA0 [28,37]. The apparent lack of regulation of the *phlA′–′lacZ* fusion by RpoS prompted us to verify that the expression of the *phlA* gene is indeed negatively controlled by RsmA. The expression of the *phlA′–′lacZ* fusion was 2- to 3-fold higher in the *rsmA* mutant CHA809 [28] than in strain CHA0 throughout growth (Fig. 2(d)). This result confirms that GacS/GacA-controlled *phlA* expression [35] involves RsmA as an intermediate in the signal transduction pathway.

3.4. RpoS does not control RsmA levels

In *E. carotovora*, RpoS positively controls the expression of RsmA [32]. We therefore monitored the amount of RsmA protein in the wild-type CHA0, in the *rpoS* mutant CHA815 [28], as well as in the *gacS* mutant CHA19 [31] and in the *gacA* mutant CHA89 [43]. However, no significant differences in RsmA levels could be detected (Fig. 3), suggesting that in strain CHA0, regulators of stationary phase-dependent phenotypes like GacS/GacA, or RpoS, do not influence RsmA concentration.

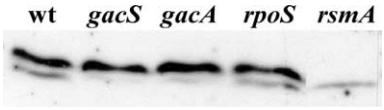

Fig. 3. RsmA levels are not affected by GacS, GacA or RpoS. An equivalent amount of cells from strains CHA0 (wt), CHA19 (*gacS*), CHA89 (*gacA*), CHA815 (*rpoS*) and CHA809 (*rsmA*) was taken from stationary phase cultures, subjected to SDS–PAGE, and analysed by Western blotting with antibodies raised against *Y. enterocolitica* RsmA [41]. The optical density of the cultures at 600 nm was 4.6–5.4 units at the moment of sampling. The band migrating below RsmA represents the RsmA homologue RsmE [48].

3.5. Oxidative stress survival is under Gac/Rsm/RpoS control

The aim here was to determine whether resistance to oxidative stress is controlled by the postulated Gac/Rsm/RpoS cascade. Thus, we assayed the survival of stationary-phase cells after exposure to hydrogen peroxide in the wild-type strain CHA0, the *gacS* mutant CHA19, the *rsmA* mutant CHA809, and the *rpoS* mutant CHA815. Resistance to H_2O_2 is caused probably by catalase(s) and/or peroxidase(s) which are under GacS/GacA control [26]. Loss of *gacS* or *rpoS* function both caused a drastic reduction in survival, whereas an *rsmA* mutation had little effect (Table 1). The sensitivity of the *gacS* mutant to hydrogen peroxide could be suppressed to wild-type levels by overexpressing either *rpoS* with pME6354 or *rsmZ* with pME6359, when comparing to the wild-type strain carrying the empty expression vectors pME6001 [37] or pME6032 [28], respectively (Table 1). Overexpression of *rsmA* in strain CHA0 with plasmid pME6073 [28] mimicked a *gacS* defect (Table 1).

Table 1
Survival of *P. fluorescens* strains following exposure to H_2O_2

Strain	Genotype	Viable counts (CFU ml^{-1}) at		Survival (%)
		$t = 0$	$t = 1$ h	
CHA0	Wild type	$6.2 \pm 0.2 \times 10^8$	$5.0 \pm 0.5 \times 10^8$	80.6
CHA815	Δ*rpoS*	$4.8 \pm 0.6 \times 10^8$	$8.7 \pm 1.6 \times 10^6$	1.8
CHA19/pME6001	Δ*gacS*	$3.0 \pm 0.1 \times 10^8$	$1.8 \pm 2.8 \times 10^5$	0.1[a]
CHA19/pME6354	Δ*gacS rpoS*$^{++}$	$3.0 \pm 0.6 \times 10^8$	$2.8 \pm 0.3 \times 10^8$	93.3
CHA19/pME6032	Δ*gacS*	$1.8 \pm 0.6 \times 10^8$	$2.3 \pm 0.3 \times 10^6$	1.3
CHA19/pME6359	Δ*gacS rsmZ*$^{++}$	$4.6 \pm 1.7 \times 10^8$	$4.7 \pm 2.6 \times 10^8$	102.2
CHA809	*rsmA*:: Ω Km^R	$7.5 \pm 1.7 \times 10^8$	$4.6 \pm 1.5 \times 10^8$	61.3
CHA0/pME6001	Wild type	$3.0 \pm 0.5 \times 10^8$	$1.1 \pm 0.9 \times 10^7$	3.8[a]
CHA0/pME6073	*rsmA*$^{++}$	$3.5 \pm 1.0 \times 10^8$	$4.7 \pm 1.0 \times 10^5$	0.1

Strains were grown to early stationary phase.
At $t = 0$, when 40 mM H_2O_2 was added, and after 1 h of exposure ($t = 1$ h), viable counts were determined in triplicate (±SD).
[a] The gentamicin resistance plasmid pME6001 was included as a control as it had a strong negative effect on viability, for unknown reasons. Plasmids pME6354 and pME6073 are pME6001 derivatives, whereas pME6359 is a derivative of the vector pME6032 [28].

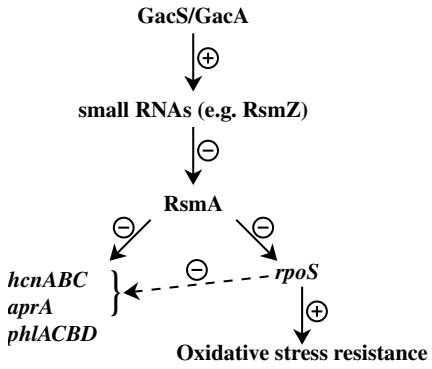

Fig. 4. Proposed pathway depicting the role of RpoS in Gac/Rsm-controlled secondary metabolism and resistance to oxidative stress in *P. fluorescens* CHA0. Signal transduction involving the GacS/GacA two-component system positively controls the transcription of small regulatory RNAs such as RsmZ and RsmY. These regulatory RNAs antagonise the RsmA-mediated post-transcriptional regulation of target genes such as the *hcn*, *apr* and *phl* operons. RpoS, being also controlled by this pathway, positively controls the resistance to oxidative stress, and negatively affects the expression of the *hcnA* and *aprA* genes, presumably indirectly by competition with RpoD (σ^{70}) for RNA polymerase core enzyme.

Together, these results support the existence of a linear GacS/GacA → RsmZ ⊣ RsmA ⊣ RpoS → oxidative stress resistance pathway (Fig. 4).

4. Discussion

The observation that in some, but not all, fluorescent pseudomonads the expression of RpoS is positively controlled by the GacS/GacA two-component system [22,24–26] incited us to investigate two aspects of the Gac regulon in the biocontrol organism *P. fluorescens* CHA0: first, the question whether the two-component system GacS/GacA and the RNA-binding protein RsmA, a key regulatory element in the Gac regulon [37], regulate RpoS expression and hence RpoS-controlled resistance to oxidative stress, and second, the question whether RpoS is an intermediate element in Gac/Rsm-controlled expression of secondary metabolism and exoenzymes.

The fact that an *rpoS′–′lacZ* fusion was poorly expressed in *gacA* background and overexpressed in an *rsmA* mutant, by comparison with the wild type (Fig. 2(a)), suggests that the Gac/Rsm cascade activates RpoS expression in *P. fluorescens* CHA0. This is in agreement with the data of Whistler et al. [24] who showed that in *gacS* or *gacA* mutants of *P. fluorescens* Pf-5 RpoS protein levels were 20–50% of those present in the wild type. Whereas the mechanism by which the Gac/Rsm cascade regulates *rpoS* expression is still unknown, the effects of this cascade could be clearly seen at the level of resistance to hydrogen peroxide (Table 1) in that both a *gacS* and an *rpoS* mutation resulted in a 50-fold decrease of viability within 1 h of incubation with hydrogen peroxide, and a similar effect occurred in an *rsmA* overexpressing background. An *rsmA*-negative mutant manifested a slight reduction (rather than an increase) in resistance to oxidative stress, suggesting that the *rsmA* mutation may have some negative side effects on the viability of the strain (Table 1). By contrast, the presence of either *rsmZ* or *rpoS* on a multi-copy plasmid entirely suppressed a *gacS* defect, confirming the model (Fig. 4) according to which *rsmZ* overexpression would relieve RsmA-mediated repression of *rpoS* translation and *rpoS* overexpression would override the *gacS* signalling defect. It will be interesting to find out whether the extensive *rpoS* 5′ leader transcript [21,23] is able to bind RsmA and other regulatory elements such as small RNAs [44].

RpoS is unlikely to be an intermediate in Gac/Rsm-dependent control of *hcnA* and *aprA* expression, as in both cases the effect of RpoS was clearly negative (Figs. 2(b) and (c)). We interpret these observations in terms of competition of different σ factors for RNA polymerase core enzyme: the house-keeping σ^{70} has the strongest affinity and σ^{38} (RpoS) has the weakest affinity for the core enzyme [45]. If, as it appears likely, the transcription from the *hcnA*, *aprA* and *phlA* promoters is essentially driven by σ^{70} RNA polymerase, an excess of σ^{38} would be expected to have a negative effect on the transcription of these genes, and this is compatible with our results as well as with observations made on HCN and Phl biosynthesis in *P. fluorescens* Pf-5 [4,24,46].

Although the Gac/Rsm cascade is conserved in many Gram-negative bacteria, the target genes regulated vary widely and so does the fine-tuning of the cascade [29,47]. For instance, RpoS positively controls RsmA synthesis in *E. carotovora*, whereas we see no measurable effect of RpoS on RsmA levels in *P. fluorescens* CHA0 (Fig. 3) but, on the contrary, we have obtained evidence for negative control of *rpoS* expression by RsmA (Fig. 2(a)). In strain CHA0, the regulatory effects of an *rsmA* mutation on the expression of target genes are less pronounced than are those of *gacS* and *gacA* mutations [37], essentially because this bacterium has a second RsmA-like regulatory protein, RsmE, whose expression is under GacA control [48]. The role of small regulatory RNAs, i.e. RsmZ [28] and RsmY [30], in the regulation of *rpoS* is currently under investigation.

Acknowledgements

We thank Paul Williams for the anti-RsmA antibodies, Karin Heurlier for her assistance in the detection of RsmA by Western blotting, and Kirsten Bang Lejbølle for the construction of pME6354. Support from the Swiss National Foundation for Scientific Research (projects 31-64048.00 and 3100A0-100180) is gratefully acknowledged.

References

[1] Loewen, P.C., Hu, B., Strutinsky, J. and Sparling, R. (1998) Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. 44, 707–717.

[2] Hengge-Aronis, R. (2002) Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. 66, 373–395.

[3] Tanaka, K. and Takahashi, H. (1994) Cloning, analysis and expression of an *rpoS* homologue gene from *Pseudomonas aeruginosa* PAO1. Gene 150, 81–85.

[4] Sarniguet, A., Kraus, J., Henkels, M.D., Muehlchen, A.M. and Loper, J.E. (1995) The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. Proc. Natl. Acad. Sci. USA 92, 12255–12259.

[5] Jørgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P. and Stewart, G.S. (1999) RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. Microbiology 145, 835–844.

[6] Kojic, M., Degrassi, G. and Venturi, V. (1999) Cloning and characterisation of the *rpoS* gene from plant growth-promoting *Pseudomonas putida* WCS358: RpoS is not involved in siderophore and homoserine lactone production. Biochim. Biophys. Acta 1489, 413–420.

[7] Suh, S.-J., Silo-Suh, L., Woods, D.E., Hasset, D.J., West, S.E.H. and Ohman, D.E. (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J. Bacteriol. 181, 3890–3897.

[8] Mukhopadhyay, S., Audia, J.P., Roy, R.N. and Schellhorn, H.E. (2000) Transcriptional induction of the conserved alternative sigma factor RpoS in *Escherichia coli* is dependent on BarA, a probable two-component regulator. Mol. Microbiol. 37, 371–381.

[9] Lange, R. and Hengge-Aronis, R. (1994) The cellular concentration of the σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev. 8, 1600–1612.

[10] Bohringer, J., Fischer, D., Mosler, G. and Hengge-Aronis, R. (1995) UDP–glucose is a potential intracellular signal molecule in the control of expression of σ^S and σ^S-dependent genes in *Escherichia coli*. J. Bacteriol. 177, 413–422.

[11] Shiba, T., Tsutsumi, K., Yano, H., Ihara, Y., Kameda, A., Tanaka, K., Takahashi, H., Munekata, M., Rao, N.N. and Kornberg, A. (1997) Inorganic polyphosphate and the induction of *rpoS* expression. Proc. Natl. Acad. Sci. USA 94, 11210–11215.

[12] Muffler, A., Traulsen, D.D., Fischer, D., Lange, R. and Hengge-Aronis, R. (1997) The RNA-binding protein HF-I plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the σ^S subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. 179, 297–300.

[13] Barth, M., Marschall, C., Muffler, A., Fischer, D. and Hengge-Aronis, R. (1995) Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^S and many σ^S-dependent genes in *Escherichia coli*. J. Bacteriol. 177, 3455–3464.

[14] Klauck, E., Bohringer, J. and Hengge-Aronis, R. (1997) The LysR-like regulator LeuO in *Escherichia coli* is involved in the translational regulation of *rpoS* by affecting the expression of the small regulatory DsrA–RNA. Mol. Microbiol. 25, 559–569.

[15] Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R. and Storz, G. (1998) The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein. EMBO J. 17, 6061–6068.

[16] Majdalani, N., Chen, S., Murrow, J., John, K.S. and Gottesman, S. (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA. Mol. Microbiol. 39, 1382–1394.

[17] Muffler, A., Barth, M., Marschall, C. and Hengge-Aronis, R. (1997) Heat shock regulation of σ^S turnover: a role for DnaK and relationship between stress responses mediated by σ^S and σ^{32} in *Escherichia coli*. J. Bacteriol. 179, 445–452.

[18] Zhou, Y., Gottesman, S., Hoskins, J.R., Maurizi, M.R. and Wickner, S. (2001) The RssB response regulator directly targets σ^S for degradation by ClpXP. Genes Dev. 15, 627–637.

[19] Ishihama, A. (2000) Functional modulation of *Escherichia coli* RNA polymerase. Ann. Rev. Microbiol. 54, 499–518.

[20] Ramos-González, M.I. and Molin, S. (1998) Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. J. Bacteriol. 180, 3421–3431.

[21] Kojic, M., Aguilar, C. and Venturi, V. (2002) TetR family member PsrA directly binds the *Pseudomonas rpoS* and *psrA* promoters. J. Bacteriol. 184, 2324–2330.

[22] Bertani, I., Sevo, M., Kojic, M. and Venturi, V. (2003) Role of GacA, LasI, RhlI, Ppk, PsrA, Vfr and ClpXP in the regulation of the stationary-phase sigma factor *rpoS*/RpoS in *Pseudomonas*. Arch. Microbiol. 180, 264–271.

[23] Venturi, V. (2003) Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different?. Mol. Microbiol. 49, 1–9.

[24] Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W. and Loper, J.E. (1998) The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 180, 6635–6641.

[25] Schmidt-Eisenlohr, H., Gast, A. and Baron, C. (2003) Inactivation of *gacS* does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. Appl. Environ. Microbiol. 69, 1817–1826.

[26] Kang, B.R., Cho, B.H., Anderson, A.J. and Kim, Y.C. (2004) The global regulator GacS of a biocontrol bacterium *Pseudomonas chlororaphis* O6 regulates transcription from the *rpoS* gene encoding a stationary-phase sigma factor and affects survival in oxidative stress. Gene 325, 137–143.

[27] Voisard, C., Bull, C.T., Keel, C., Laville, J., Maurhofer, M., Schnider, M., Défago, G. and Haas, D. (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches In: Molecular Ecology of Rhizosphere Microorganisms (O'Gara, F., Dowling, F.D.N. and Boesten, B., Eds.), pp. 67–89. VCH Verlagsgesellschaft mbH, Germany.

[28] Heeb, S., Blumer, C. and Haas, D. (2002) Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J. Bacteriol. 184, 1046–1056.

[29] Heeb, S. and Haas, D. (2001) Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. Mol. Plant–Microbe Interact. 14, 1351–1363.

[30] Valverde, C., Heeb, S., Keel, C. and Haas, D. (2003) RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. Mol. Microbiol. 50, 1361–1379.

[31] Zuber, S., Carruthers, F., Keel, C., Mattart, A., Blumer, C., Pessi, G., Gigot-Bonnefoy, C., Schnider-Keel, U., Heeb, S., Reimmann, C. and Haas, D. (2003) GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. Mol. Plant–Microbe Interact. 16, 634–644.

[32] Mukherjee, A., Cui, Y., Ma, W., Liu, Y., Ishihama, A., Eisenstark, A. and Chatterjee, A.K. (1998) RpoS (σ^S) controls expression of *rsmA*, a global regulator of secondary metabolites, harpin, and extracellular proteins in *Erwinia carotovora*. J. Bacteriol. 180, 3629–3634.

[33] Castañeda, M., Sánchez, J., Moreno, S., Núñez, C. and Espín, G. (2001) The global regulators GacA and σ^S form part of a cascade

that controls alginate production in *Azotobacter vinelandii*. J. Bacteriol. 183, 6787–6793.
[34] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[35] Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Défago, G., Haas, D. and Keel, C. (2000) Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J. Bacteriol. 182, 1215–1225.
[36] Del Sal, G., Manfioletti, G. and Schneider, C. (1988) Del A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res. 16, 9878.
[37] Blumer, C., Heeb, S., Pessi, G. and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. USA 96, 14073–14078.
[38] Minton, N.P. (1984) Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene 31, 269–273.
[39] Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[40] Schagger, H. and von Jagow, G. (1987) Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379.
[41] Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T.G., Cámara, M., Haas, D. and Williams, P. (2001) The global post-transcriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. J. Bacteriol. 183, 6676–6683.
[42] Fujita, M., Tanaka, K., Takahashi, H. and Amemura, A. (1994) Transcription of the principal sigma-factor genes, *rpoD* and *rpoS*, in *Pseudomonas aeruginosa* is controlled according to the growth phase. Mol. Microbiol. 13, 1071–1077.
[43] Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G. and Haas, D. (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. USA 89, 1562–1566.
[44] Repoila, F., Majdalani, N. and Gottesman, S. (2003) Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm. Mol. Microbiol. 48, 855–861.
[45] Maeda, H., Fujita, N. and Ishihama, A. (2000) Competition among seven *Escherichia coli* sigma subunits: relative binding affinities to the core RNA polymerase. Nucleic Acids Res. 28, 3497–3503.
[46] Schnider, U., Keel, C., Blumer, C., Troxler, J., Défago, G. and Haas, D. (1995) Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. 177, 5387–5392.
[47] Heeb, S., Heurlier, K., Valverde, C., Cámara, M., Haas, D. and Williams, P. (2004) Post-transcriptional regulation in *Pseudomonas* spp. via the Gac/Rsm regulatory network (Ramos, J.-L., Ed.), Pseudomonas, Vol. 2, pp. 239–255. Kluwer Academic Publishers, New York.
[48] Reimmann, C., Valverde, C., Kay, E. and Haas, D. (2005) Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. J. Bacteriol. 187, 276–285.