RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*

Frieda Jørgensen,¹† Marc Bally,² Virginie Chapon-Herve,² Gerard Michel,² Andrée Lazdunski,² Paul Williams³ and G. S. A. B. Stewart^{1,3}

Author for correspondence: Frieda Jørgensen. Tel: +44 1392 412853. Fax: +44 1392 412835.
e-mail: frieda.jorgensen@mailexcite.com

¹ School of Biological Sciences, Food Sciences Division, University of Nottingham, Sutton Bonington Campus, LE12 5RD, UK

² Laboratoire d'Ingénierie et Dynamique des Systèmes Membranaires, Centre National de la Recherche Scientifique, 31 chemin Joseph Aiguier, 13402 Marseilles Cedex 20, France

³ School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

***Pseudomonas aeruginosa* is able to persist during feast and famine in many different environments including soil, water, plants, animals and humans. The alternative sigma factor encoded by the *rpoS* gene is known to be important for survival under stressful conditions in several other bacterial species. To determine if the *P. aeruginosa* RpoS protein plays a similar role in stationary-phase-mediated resistance, an *rpoS* mutant was constructed and survival during exposure to hydrogen peroxide, high temperature, hyperosmolarity, low pH and ethanol was investigated. Disruption of the *rpoS* gene resulted in a two- to threefold increase in the rate of kill of stationary-phase cells. The *rpoS* mutant also survived less well than the parental strain during the initial phase of carbon or phosphate-carbon starvation. However, after 25 d starvation the remaining population of culturable cells was not significantly different. Stationary-phase cells of the RpoS-negative strain were much more stress resistant than exponentially growing RpoS-positive cells, suggesting that factors other than the RpoS protein must be associated with stationary-phase stress tolerance in *P. aeruginosa*. Comparison of two-dimensional PAGE of the *rpoS* mutant and the parental strain showed four major modifications of protein patterns associated with the *rpoS* mutation.**

Keywords: RpoS, stress tolerance, stationary phase, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an adaptable opportunistic human pathogen which can be found in many different environments including soil, water, plants, animals and humans. To successfully persist in a changing environment a micro-organism must sense such change and react appropriately. The adaptation to stressful and growth-limiting conditions encountered in host tissue, as well as in natural environments, may rely upon mechanisms common to the response observed when bacteria in culture are depleted of nutrients or exposed to other stresses (Kolter *et al.*, 1993). Analysis of the response of different bacterial species to such unfavourable environmental conditions often coincides with the induction of multiple stress tolerance and production of virulence factors (Huisman *et al.*, 1996; Hengge-Aronis, 1996; Mahan *et al.*, 1996). It is because the ability to

mount such a response will help the successful pathogen to persist in the environment and evade host defence systems that much recent work has focused on elucidating the molecular mechanisms governing global stress tolerance.

Studies in *Escherichia coli* have demonstrated that the stress response is very complex, consisting of changes in global gene expression including general as well as stress-specific components, and is accompanied by physiological and morphological changes. It is now clear that the *rpoS*-encoded σ^S subunit of RNA polymerase is an important regulator of the general stress response in *E. coli* (Muffler *et al.*, 1997). Initially, the transcription of the *rpoS* gene was shown to increase during entry into stationary phase (Lange & Hengge-Aronis, 1991) but the cellular level of the protein has now been demonstrated to also increase upon exposure to hyperosmotic stress (Muffler *et al.*, 1996), acid stress (Lee *et al.*, 1995), exposure to low temperature (Sledjeski *et al.*, 1996) as well as heat shock (Muffler *et al.*, 1997). Consistent with a role for the RpoS protein in con-

† Present address: PHLS Food Microbiology Research Unit, Church Lane, Heavitree, Exeter EX2 5AD, UK.

tributing to multiple stress tolerance, stationary-phase *rpoS* mutants of *E. coli* show increased sensitivity to starvation, high osmolarity, heat stress, hydrogen peroxide and acid stress (McCann *et al.*, 1991; Small *et al.*, 1994). *rpoS* gene homologues have been identified in other bacteria including *Salmonella* spp., *Klebsiella pneumoniae*, *Shigella flexneri*, *Yersinia enterocolitica* and *Vibrio cholerae* (Fang *et al.*, 1992; Loewen & Hengge-Aronis, 1994; Small *et al.*, 1994; Badger & Miller, 1995; Yildiz & Schoolnik, 1998). In these bacteria the RpoS homologues play a similar physiological role to that in *E. coli*. Moreover, a direct requirement of *rpoS* for virulence in orally infected mice has been demonstrated in *Salmonella typhimurium* mainly due to activation of the plasmid-encoded *spv* genes but *rpoS* regulated chromosomally encoded genes also play a contributory role (Fang *et al.*, 1992). *rpoS* mutants of *Y. enterocolitica* and *V. cholerae* were, however, not attenuated for virulence in mice but a functional *rpoS* gene was required for production of haemagglutinin/protease in *V. cholerae* and full expression of the Yst enterotoxin in *Y. enterocolitica* (Yildiz & Schoolnik, 1998; Badger & Miller, 1995; Iriarte *et al.*, 1995). The existence of RpoS homologues in bacteria such as *Sinorhizobium* (*Rhizobium*) *meliloti* and *Acetobacter methanolicus* has been inferred from the experiments carried out by Miksch & Dobrowolski (1995), suggesting that RpoS-mediated regulatory mechanisms are conserved in more distantly related Gram-negative species.

P. aeruginosa possesses an RpoS homologue (Tanaka & Takahashi, 1994). RpoS levels in *P. aeruginosa* increase upon entry into stationary phase as observed in other Gram-negative bacteria (Fujita *et al.*, 1994). Compared with *E. coli* relatively little is known about the mechanisms of the regulation of the RpoS protein in *P. aeruginosa*. However, the cell-density-dependent signalling mechanism termed quorum sensing was recently linked to the expression of the *rpoS* gene in this bacterium (Latifi *et al.*, 1996). Two quorum sensing regulons have been identified in *P. aeruginosa* in which the LuxR homologues LasR and RhlR are activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL), respectively (Latifi *et al.*, 1996). These two circuits regulate a wide array of exoproducts (proteases, toxin and secondary metabolites) which are essential for virulence (Latifi *et al.*, 1995). Expression of the *rpoS* gene was abolished in a *P. aeruginosa lasR* mutant and in the BHL-negative mutant PANO67 (Latifi *et al.*, 1996). In *E. coli* an *rpoS-lacZ* fusion was regulated directly by RhlR/BHL (Latifi *et al.*, 1996). It is also plausible that additional factors participate in the regulation of RpoS synthesis. Recently, Kim *et al.* (1998) identified a link between regulation of exopolysaccharide alginate synthesis, cellular levels of ppGpp and survival of *P. aeruginosa* in stationary phase. It was suggested that this latter effect might be related to the expression of the RpoS protein in response to high levels of ppGpp, a modulator of the synthesis of the RpoS protein in *E. coli*

(Gentry *et al.*, 1993). *Pseudomonas* spp. are known to develop stress tolerance and increase their production of antibiotics upon entry into stationary phase (Givskov *et al.*, 1994; Klotz & Hutcheson, 1992; Sarniguet *et al.*, 1995). Growth-phase-dependent expression of azurin, a protein required for the cellular response of *P. aeruginosa* to redox stress, involves expression of the *rpoS* gene (Vijgenboom *et al.*, 1997). In a *Pseudomonas fluorescens rpoS* mutant antibiotic production was affected and survival during oxidative and osmotic stress and on plant surfaces was decreased (Sarniguet *et al.*, 1995).

In the present study we report on the role of the RpoS protein in conferring stress tolerance in *P. aeruginosa* towards a variety of environmental stresses.

METHODS

Bacterial strains and media. *P. aeruginosa* wild-type strain PAO1 (Holloway collection) and its *rpoS*-null derivative PAOS (this study) were grown at 37 °C with shaking, at 200 r.p.m. in LB broth, Lennox (Difco) or in a chemically defined medium (CDM) containing 24 mM glucose, 48 mM (NH₄)₂SO₄, 3.84 mM K₂HPO₄·3H₂O, 0.74 mM KCl, 0.6 mM NaCl, 0.48 mM MgSO₄·7H₂O, 60 mM MOPS and 0.01 mM FeSO₄·7H₂O (Kadurugamuwa *et al.*, 1987). The omission of either glucose (CDM-C) or glucose and K₂HPO₄·3H₂O (CDM-PC) from CDM provided carbon and carbon-phosphate starvation media, respectively. Unless otherwise stated all chemicals were purchased from BDH. PAOS was grown in the presence of 300 µg carbenicillin ml⁻¹. Growth of the cultures was measured as OD₄₇₀ with a Gallenkamp Visi-Spec spectrophotometer. A starter culture (20 ml inoculated with a single colony and incubated for 16 h) was used to initiate the exponential and stationary-phase cultures used in the stress tolerance assays. Exponential-phase cultures were obtained by diluting the starter culture 1:50 in fresh media. Cells were then grown to an OD₄₇₀ of 0.7, diluted again to an OD₄₇₀ of 0.1 and finally grown to mid-exponential phase (corresponding to 4–6 × 10⁸ cells or OD₄₇₀ = 0.7). Stationary-phase cultures were obtained by diluting the starter culture to an OD₄₇₀ of 0.05 and then incubating for 24 h reaching a final cell density of 8–9 × 10⁹.

Plasmids and DNA procedures. The suicide plasmid pUR' was constructed by cloning of a 0.55 kb *HincII*–*PstI* DNA fragment internal to the *rpoS* gene (Tanaka & Takahashi, 1994) into pUC18. The plasmid pLFRS was obtained by insertion of a 1.8 kb *KpnI*–*HindIII* fragment from pDB18R containing a functional *rpoS* gene (Tanaka & Takahashi, 1994) into the broad-host-range vector pLAFR3 (Friedman *et al.*, 1982). Plasmid DNA was prepared and digested with restriction enzymes according to techniques described by Sambrook *et al.* (1989). Recombinant plasmids were transferred to *P. aeruginosa* by electroporation as previously described by Smith & Iglewski (1989). Chromosomal DNA preparations were carried out according to the protocol established by Chen & Kuo (1993). Hybridization probes were prepared with DNA fragments extracted from agarose gels (Sambrook *et al.*, 1989), labelled with digoxigenin and detected by using chemiluminescence (DIG Luminescent Detection kit; Boehringer Mannheim).

Construction of *rpoS* mutant strain PAOS. The suicide plasmid pUR' was introduced into PAO1 by electroporation and

Fig. 1. Construction of an *rpoS* mutant of *P. aeruginosa* PAO1. (a) Homologous recombination between the PAO1 chromosome (*rpoS*-positive; bottom) and the suicide plasmid pUR' carrying a 0.55 kb *HincII*-*PstI* fragment internal to the *rpoS* gene. (b) Partial restriction map of the chromosome of strain PAOS, in which the *rpoS* gene is disrupted by the insertion of the pUR' suicide plasmid. Vector sequences integrated into the chromosome are shown by a zig-zag line. (c) Southern blot illustrating the insertion of pUR' in the PAOS chromosome. Chromosomal DNA of PAO1 (lanes 1 and 2) and PAOS (lanes 3 and 4) was digested with *Bam*HI (lanes 1 and 3) or *Bam*HI and *Eco*RI (lanes 2 and 4). The probe was a 1.8 kb *Kpn*I-*Hind*III fragment (derived from pDB18R; Tanaka & Takahashi, 1994) carrying the entire *rpoS* gene. DNA size markers (in kb; Bethesda Research Laboratories) are indicated to the right. (d) Western blot detection of the RpoS protein in cell lysates of PAO1 (lane 1), PAOS (lane 2) and PAOS(pLFRS) (lane 3). The RpoS protein is shown by an arrow. Proteins from equivalent amounts of cells grown until early stationary phase were separated on an 11% SDS-PAGE gel before immunodetection with anti-RpoS antibodies. Molecular mass markers are shown on the left (in kDa).

transformed colonies were screened on plates containing 300 µg carbenicillin ml⁻¹. Correct integration was confirmed by Southern blot analysis (Sambrook *et al.*, 1989). Immunodetection of the RpoS protein by Western blot analysis was performed as described elsewhere (Latifi *et al.*, 1995).

Two-dimensional gel electrophoresis. Cells were grown at 37 °C in MNP medium at 180 r.p.m. (Holloway, 1955). Late-exponential-phase cells were labelled with 1.8 MBq [³⁵S]methionine ml⁻¹ for 1 h. Labelling was stopped by the addition of 1 mM non-radioactive methionine on ice, then the cells were centrifuged (10000 g, 5 min, 4 °C) and washed with cold 10 mM Tris/HCl (pH 7.5) containing 10% (w/v) sucrose. Proteins were solubilized in the solubilization buffer of O'Farrell (1975) after several freeze-thawing treatments and separated by two-dimensional gel electrophoresis according to O'Farrell (1975). The pH gradient in the focusing gel was established using 2% (w/v) Ampholines (LKB) containing 1.6% (w/v) Ampholines (pH 5–7) and 0.4% (w/v) Ampholines (pH 3.5–10). Isoelectric focusing gels were loaded with approximately 100 µg protein and electrophoresis was carried out at 400 V for 18 h (7200 V h). In the second dimension,

proteins were separated by SDS-PAGE (12%, w/v, acrylamide gels) according to Laemmli (1970).

Measurement of resistance to hydrogen peroxide, high temperature, low pH, ethanol and high salt. *P. aeruginosa* cultures were assayed for the ability to survive oxidative stress (50 mM or 10 mM H₂O₂), heat stress (53 °C), osmotic stress (3 M NaCl), pH stress (media acidified to pH 3 using HCl) and ethanol stress (10%, v/v, ethanol) in LB and CDM. The stress media were pre-warmed to 37 °C or 53 °C (heat stress assay only) and were all prepared immediately prior to the measurement of stress tolerance. Stationary-phase or exponential-phase cultures were diluted in the respective growth medium so that by adding 10 µl of this diluted culture to the pre-warmed stress medium, a final cell density of 1 × 10⁷ c.f.u. ml⁻¹ was obtained. Less than 5 min elapsed from the initial dilution of the cultures until exposure to the stress. Samples were removed immediately and, at timed intervals, diluted in their respective growth medium and plated onto Tryptic Soy Agar (TSA; Difco). Colonies were counted after 2 d at 37 °C. When assaying PAOS cultures, carbenicillin (300 µg ml⁻¹) was added to the stress medium and to the TSA plates. Each stress

tolerance assay was performed in duplicate or triplicate and performed at least twice unless otherwise stated.

Measurement of starvation survival rates. Exponential-phase cells grown in CDM prepared as described above were harvested by centrifugation (8000 g, 10 min), washed twice in CDM-C or CDM-PC and then suspended in the respective starvation medium to give a final cell density of $5\text{--}7 \times 10^7$ c.f.u. ml⁻¹. These cultures were held at 37 °C at 200 r.p.m. for up to 33 d. Samples were taken in duplicate at intervals, diluted in LB, plated onto TSA and counted after 2 d at 37 °C.

RESULTS

Construction of an *rpoS*-null mutant

The *rpoS* gene in PAO1 was mutated by insertional inactivation as described in Methods and illustrated in Fig. 1(a, b). After electrotransformation of PAO1 by the suicide plasmid pUR', a relatively large number of carbenicillin-resistant colonies were obtained. One of these clones, designated PAOS, was further examined. The possible disruption of the *rpoS* gene, resulting from the homologous recombination between pUR' and the PAO1 chromosome, was verified by Southern blot analysis. The chromosomal DNA extracted from PAOS and its parental strain PAO1 was digested with *Bam*HI to establish whether a new chromosomal 3.3 kb *Bam*HI fragment containing the pUR' sequences was detected (Fig. 1c, lanes 1 and 3). A single new band of the expected size was seen to hybridize with the *Kpn*I-*Hind*III *rpoS* gene probe, while the 1.9 kb and 4.5 kb *Bam*HI fragments corresponding to sequences extending upstream and downstream of the *rpoS* gene were unchanged between the two strains. When the chromosomal DNA preparations were cut with *Bam*HI and *Eco*RI (Fig. 1c, lanes 2 and 4), a new band of 3 kb was present which corresponds to the pUR' vector insertion into *rpoS* sequences. The 1.9 kb *Bam*HI fragment and

the 1 kb *Bam*HI-*Eco*RI fragment, encompassing the 5' and 3' extremities of the *rpoS* gene, respectively, were unchanged between the parental and the mutant strain. The absence of RpoS protein in the mutant was confirmed by Western blot analysis (Fig. 1d). Introduction of pLFRS, a pLAFR3 derivative carrying the *rpoS* gene, into the PAOS mutant strain re-established the synthesis of the RpoS protein (Fig. 1d). These analyses permitted us to conclude that the insertional mutation occurred within the *rpoS* gene in the PAOS strain.

Stress resistance of stationary-phase cells grown in complex and chemically defined media is affected by an altered *rpoS* allele

Survival of stationary-phase PAOS and wild-type PAO1 strains of *P. aeruginosa* after exposure to hydrogen peroxide, heat stress, high osmolarity, low pH and ethanol was examined. The PAOS strain was significantly more sensitive than the wild-type strain to all of the stresses examined both when grown in a complex medium (LB) and in a chemically defined medium (CDM) (Table 1). When survival was compared by single time point analysis, the number of wild-type PAO1 strain survivors was 5- to 80-fold greater than the number of PAOS strain survivors depending on the stress factor and medium (Table 1). Assessing the sensitivity of stationary-phase PAO1 and PAOS cells grown in LB to the same stresses by comparing the survival rate (Figs 2 and 3) also showed a clear difference (two- to threefold).

The medium the cultures were grown and challenged in affected the sensitivity of the cells to stress. Cells in CDM were more sensitive to stress than those grown and challenged in LB. As shown in Table 1, 5 min at

Table 1. Survival of stationary-phase cells of *P. aeruginosa* PAO1 (wild-type) and PAOS (*rpoS*-null mutant) upon exposure to environmental stresses

Values were calculated as $(\log_{10} a) - (\log_{10} b)$, where *a* represents the number of culturable cells before exposure to stress and *b* represents the number of culturable cells after exposure to stress. The numbers in parentheses are the standard error of the mean.

Stress*	$\log_{10}$ reduction in cell numbers after exposure to the indicated stress					
	LB			CDM		
	PAO1	PAOS	<i>P</i> value	PAO1	PAOS	<i>P</i> value
H ₂ O ₂ (50 mM)	1.50 (0.120)	2.8 (0.22)	<0.001	1.10 (0.180)	3.0 (0.340)	<0.001
Heat (53 °C)	0.44 (0.040)	2.2 (0.36)	<0.01	1.40 (0.110)	2.3 (0.120)	<0.001
NaCl (3 M)	0.85 (0.059)	2.3 (0.17)	<0.001	0.72 (0.033)	2.0 (0.140)	<0.01
pH 3 (HCl)	2.60 (0.180)	4.0 (0.19)	<0.001	0.89 (0.150)	1.6 (0.048)	<0.05
Ethanol (10 %, v/v)	0.89 (0.160)	1.6 (0.29)	<0.01	1.60 (0.056)	2.9 (0.140)	<0.001

* Exposure to H₂O₂ was maintained for 25 min in LB and 10 min in CDM; exposure to 53 °C was maintained for 25 min in LB and 5 min in CDM; exposure to NaCl was maintained for 12 h in LB and 4 h in CDM; exposure to pH 3 was maintained for 20 min in LB and 10 min in CDM; exposure to ethanol was maintained for 2.5 h in LB and 1.75 h in CDM.

Fig. 2. Survival of stationary-phase (filled symbols) and exponential-phase (open symbols) cells of *P. aeruginosa* PAO1 (squares) and PAOS (circles) in LB with 50 mM H₂O₂ (a) or LB with 10 mM H₂O₂ (exponential-phase cells only) (b). Values for stationary-phase cells represent means of at least two experiments each performed in duplicate. Error bars represent standard errors of the means. Values for exponential-phase cells are from one experiment performed in duplicate. All cultures were grown in LB.

53 °C resulted in a decrease of 1.4 log₁₀(c.f.u. ml⁻¹) for cells grown and challenged in CDM whereas in LB, 25 min at 53 °C only resulted in a decrease of 0.44 log₁₀(c.f.u. ml⁻¹). It is well known that measured resistance can be affected by the growth and challenge media used (Hansen & Riemann, 1963).

The RpoS-negative strain survived less well than the parental PAO1 strain during the initial phase of carbon starvation (Fig. 4a) and phosphate-carbon starvation (Fig. 4b). However, after 25 d starvation the remaining population of culturable cells was not dramatically different for strain PAOS (mean percentage survival: 1.4% and 2.3% in CDM-C and CDM-CP, respectively) compared with strain PAO1 (mean percentage survival: 1.8% and 2.1% in CDM-C and CDM-CP, respectively) (Fig. 4).

Fig. 3. Survival of stationary-phase (filled symbols) and exponential-phase (open symbols) cells of *P. aeruginosa* PAO1 (squares) and PAOS (circles) in LB, at 53 °C (a), with 3 M NaCl (b), at pH 3.0 (c) and in 10% (v/v) ethanol (d). Values for stationary-phase cells represent means of at least two experiments each performed in duplicate. Error bars represent standard errors of the means. Values for exponential-phase cells are from one experiment performed in duplicate. All cultures were grown in LB.

Fig. 4. Long-term survival of *P. aeruginosa* PAO1 (■) and PAOS (○) cells during carbon starvation (a) and phosphate-carbon starvation (b). Values represent duplicate samples from a typical experiment and each experiment was done twice. All cultures were grown in CDM.

Relative contribution of the RpoS protein to survival in exponential-phase cultures

Comparison of the survival rates of exponential-phase PAO1 and PAOS cells did not reveal significant differences (Figs 2 and 3). However, stationary-phase RpoS-negative *P. aeruginosa* was markedly more stress resistant than exponential-phase RpoS-positive cultures (Figs 2 and 3).

Effects of inactivation of the *rpoS* gene on global gene expression in *P. aeruginosa*

The *rpoS* mutant PAOS and its isogenic parent PAO1, grown in minimal medium, were pulse-labelled with [³⁵S]methionine in the exponential- to stationary-phase transition and the proteins were separated by two-dimensional gel electrophoresis (Fig. 5). Comparison of the two-dimensional PAGE autoradiograms showed that the synthesis of four proteins was reproducibly modified between the two strains. Protein C18 (18 kDa) was absent in the PAOS strain while C32 (32 kDa) was

not detected in the parental strain. Furthermore, the synthesis of two other proteins, B19 (19 kDa) and D16 (16 kDa), was decreased in PAOS compared to PAO1. Other proteins were also shown to be differentially expressed in the two strains. However, these modifications in the two-dimensional patterns were found to fluctuate between several experiments, suggesting that expression of the corresponding proteins could be determined by additional factors, possibly independent of *rpoS* gene functionality.

DISCUSSION

Stationary-phase bacterial cells possess complex mechanisms which can increase their potential to survive following exposure to diverse stress conditions. A regulator of central importance for the development of the resistant state during entry into stationary phase in *E. coli* is the alternative sigma factor RpoS (Hengge-Aronis, 1996). In this work the significance of the RpoS protein for stress resistance and protein expression in stationary-phase cells of *P. aeruginosa* was assessed.

Disruption of the *P. aeruginosa* PAO1 *rpoS* gene resulted in a two- to threefold increase in the rate of kill of stationary-phase cells following exposure to heat, low pH, high osmolarity, hydrogen peroxide and ethanol. The extent, however, of the increased sensitivity in the RpoS-negative stationary-phase cells was not as pronounced as in studies with *E. coli*. This was particularly the case for survival during oxidative, osmotic and heat stress, where, depending on the strain, growth medium and challenge conditions, 6- to 30-fold differences in the survival rate between RpoS-positive and RpoS-negative stationary-phase *E. coli* cells have been reported (Lange & Hengge-Aronis, 1991; Hengge-Aronis *et al.*, 1991; McCann *et al.*, 1991; Cheville *et al.*, 1996). However, it is important to note that various environmental factors have been demonstrated to have a profound influence on the extent of the requirement for RpoS protein for stationary-phase resistance in other bacterial species. The ability of a *Y. enterocolitica* *rpoS* mutant to survive a variety of stresses was affected at 37 °C but not at 26 °C (Badger & Miller, 1995). The degree of requirement for RpoS protein in *Sh. flexneri* for stress resistance was also shown to depend on environmental factors such as pH or anaerobiosis (Small *et al.*, 1994). The survival assays performed with carbon- and carbon-phosphate-starved *P. aeruginosa* cells indicate that the effect of the RpoS protein is only transient in such conditions. One possible explanation could be that other resistance mechanisms may prevail on RpoS-dependent stress tolerance systems in aged *P. aeruginosa* cultures. A similar transient effect was not observed in starvation studies in *E. coli* but in those experiments starvation survival was not studied for as long a period of time as in this work (McCann *et al.*, 1991; Lange & Hengge-Aronis, 1991).

Stationary-phase cells of *E. coli* and *Sh. flexneri* *rpoS* mutants have been shown to be more sensitive to acid

Fig. 5. Two-dimensional protein maps of *P. aeruginosa* PAOS (a) and PAO1 (b) strains. Cells were grown in minimal medium and labelled at late-exponential phase with [35 S]methionine (1.8 MBq ml^{-1}) for 1 h. Proteins were separated by two-dimensional PAGE and detected by autoradiography. Modifications of the protein pattern are indicated by arrows. Proteins were designated by an alphanumeric nomenclature. The letter corresponds to a pH_i zone and the number to an approximate molecular mass. Positions of the molecular mass standards are indicated on the right (a).

stress than their exponential-phase parent strains (Small *et al.*, 1994). Similar results have been observed with respect to the sensitivity to oxidative, osmotic and heat stress, e.g. starved cultures of an *E. coli* *rpoS* mutant were more sensitive than the exponential-phase parental strain (McCann *et al.*, 1991). Stationary-phase RpoS-negative cells of *P. aeruginosa*, however, were much more resistant than exponentially growing RpoS-positive cells, suggesting that factors other than the RpoS

protein which are associated with entry into stationary phase contribute to stationary-phase stress tolerance in *P. aeruginosa*. In *E. coli* and *Sal. typhimurium* *pcm*, *surE*, *mcbA*, *osmC* and *slyA* are expressed in an *rpoS*-independent manner during entry into stationary phase (Li *et al.*, 1997; Bohannon *et al.*, 1991; Gordia & Gutierrez, 1996; Buchmeier *et al.*, 1997). Some of these genes have also been linked to protection against oxidative stress (*slyA*) and osmotic stress (*osmC*) in

stationary-phase cells (Gordia & Gutierrez, 1996; Buchmeier *et al.*, 1997).

Much of our understanding of the underlying molecular changes resulting in the dramatic increase in general stress resistance when exponentially growing cells enter stationary phase has been formed on the basis of studies in *E. coli*. Several genes known to be RpoS-dependent in *E. coli* encode proteins which have been shown to be important for the resistance of stationary-phase cultures to environmental stress, e.g. *dps* (protecting DNA), *katE* (catalase), *otsA/otsB* (general stress protectant) and *xthA* (DNA repair) (Hengge-Aronis, 1996). Some of the protective mechanisms employed by *Pseudomonas* to survive stress are similar to the mechanisms found in *E. coli*, e.g. the synthesis of antioxidant enzymes such as superoxide dismutases and catalases to combat oxidative stress and the accumulation of compatible solutes to survive high osmolarity environments (Brown *et al.*, 1995; Dsouzaault *et al.*, 1993). The *sodA*-gene-encoded superoxide dismutase in *P. aeruginosa* is mainly expressed during the stationary phase of growth as in *E. coli* and has been shown to be important for oxidative stress resistance (Hassett *et al.*, 1993; Polack *et al.*, 1996; Nyström *et al.*, 1996). The *P. aeruginosa* *kato* and *kata* genes which encode catalases have been shown to be required for optimal resistance when exposed to hydrogen peroxide (Brown *et al.*, 1995). *KatB* is inducible by hydrogen peroxide in a manner similar to HPI (*KatG*) in *E. coli*, whereas *kata* is transcribed throughout growth in contrast to the RpoS-mediated growth-phase-dependent expression of HPII (*KatE*) in *E. coli* (Loewen *et al.*, 1985; Storz *et al.*, 1990; Mulvey *et al.*, 1988). Differences in the regulation of genes involved in stationary-phase resistance may help explain some of the variation observed with respect to the RpoS-dependency of stationary-phase resistance among different species. In this respect, it is worth noting that although a number of proteins were affected by a disruption of the *rpoS* gene in *P. aeruginosa* only four proteins (one absent, one elevated, two decreased) showed a consistent difference, which appears to be a less dramatic change when compared to similar studies performed in *E. coli* (32 absent or reduced; McCann *et al.*, 1991) or *Sh. flexneri* (27 absent or reduced, two elevated; Waterman & Small, 1996).

Apart from its importance in stress and starvation survival, it is possible that the RpoS protein may have a role in *P. aeruginosa* virulence. There are several examples of specific roles for alternative sigma factors in microbial pathogenesis. In *P. aeruginosa* biogenesis of flagella is under the control of the specific alternative sigma factor *FlaA*, and flagellar motility is required for virulence (Starnbach & Lory, 1992; Drake & Montie, 1988). Another sigma factor, RpoN, is involved in the expression of pili which are also required for full virulence of the organisms (Ishimoto & Lory, 1989). *AlgU* (also known as *AlgT*; Goldberg *et al.*, 1993), a homologue of the σ^{12} factor in *E. coli*, controls conversion to a mucoid phenotype in *P. aeruginosa* but is also important for heat resistance (Boucher *et al.*, 1996).

Given that the *rpoS* gene is regulated in *P. aeruginosa* via quorum sensing (Latifi *et al.*, 1996), it is possible that the RpoS protein plays a role in co-ordinating the cell-density-dependent expression of virulence determinants. In this respect, it is worth noting that the *P. aeruginosa* cytotoxic lectins PA-I and PA-II (Avichezer *et al.*, 1992) which are regulated via the RpoS protein are not produced in mutants defective in the production of N-acylhomoserine lactones (unpublished). Whether other *P. aeruginosa* virulence determinants are co-ordinately regulated via quorum sensing and RpoS protein remains to be established.

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