Role of the alternative sigma factors $\sigma^E$ and $\sigma^S$ in survival of *Salmonella enterica* serovar Typhimurium during starvation, refrigeration and osmotic shock

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The ability of *Salmonella enterica* serovar Typhimurium to survive environmental stress requires specific, coordinated, responses, which induce resistance to the stress condition. This study investigated the relative contribution of $\sigma^E$ and $\sigma^S$, the sigma factors regulating extracytoplasmic and general stress response functions, respectively, to survival at low temperature and also in media of differing osmotic strength, conditions relevant to food preservation. To determine if low-temperature storage is a signal for $\sigma^E$- and $\sigma^S$-mediated survival, the ability of *S*. Typhimurium rpoE, rpoS and rpoE/rpoS mutants to survive in a saline starvation-survival model at a refrigeration temperature (4.5 °C) was examined. Under these conditions, the rpoE mutant was significantly ($P < 0.05$) compromised compared to the parent and to an rpoS mutant. The double mutant in rpoE and rpoS displayed a cumulative defect in survival. In hyperosmotic environments (low $a_w$) containing 6 % NaCl and at refrigeration temperature, both sigma factors were important for maximum survival but $\sigma^S$ played the dominant role. Analysis of the metabolic activity of starved populations at 4.5 and 37 °C revealed significantly ($P < 0.001$) elevated electron-transport system activity in mutants in rpoE and rpoS, indicating a role for $\sigma^E$- and $\sigma^S$-regulated genes in maintaining energy homeostasis. Together these data demonstrate that $\sigma^E$ and $\sigma^S$ are important for survival of *S*. Typhimurium in conditions encountered during food processing and that the relative contribution of $\sigma^E$ and $\sigma^S$ is critically dependent on the precise nature of the stress.

INTRODUCTION

Low-temperature storage is an important method of controlling microbial growth in foods and it is essential to understand how food-borne pathogens adapt to and survive under such conditions. Many studies have focused on the responses of bacteria to cold-shock and not to prolonged storage at refrigeration temperatures. The harmful effects of exposure to low temperature, such as inefficient protein folding and hampered ribosome function, are well documented (Phadtare, 2004). A number of cold-shock proteins are induced to counteract these effects by inhibiting the formation of RNA secondary structures, which reduce the efficiency of transcription and translation (Phadtare, 2004). However, in contrast to short-term cold-shock, much less is known about bacterial responses to prolonged storage at refrigeration temperatures such as those that can occur in the food chain. We have previously observed survival of different *Salmonella enterica* serovars over many months at refrigeration temperature, and many years at ambient temperature (unpublished data).

Cold-shock activates the stress-response regulons in *E. coli* and *Salmonella enterica* serovar Typhimurium (S. Typhimurium) controlled by the alternative sigma factors RpoE ($\sigma^E$) and RpoS ($\sigma^S$) (Loewen & Hengge-Aronis, 1994; Munro et al., 1995; Kandror et al., 2002; Miticka et al., 2003; Polissi et al., 2003). These studies suggest that $\sigma^E$ and $\sigma^S$ may be important for survival of S. Typhimurium at low temperature, but this has not been investigated.

Abbreviations: ETS, electron-transport system; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride.
Another stress relevant to food processing known to activate expression of both $\sigma^E$ and $\sigma^S$ dependent genes in *E. coli* and *S. Typhimurium* is hyperosmotic shock (Hengge-Aronis *et al.*, 1993; Hengge-Aronis, 1996; Bianchi & Baneyx, 1999; Miticka *et al.*, 2003; Balaji *et al.*, 2005). However, again there has been little or no work on the role of $\sigma^E$ and $\sigma^S$ on the growth/survival of *S. enterica* in hyperosmotic environments.

The ability to survive in simple solutions with low or no nutritive value, such as water or saline, is also likely to be important for survival of *Salmonella* in the environment between hosts and during food processing. Starvation for certain nutrients renders *S. Typhimurium* more resilient than nutrient-replete cells to a variety of stresses (the starvation stress response) and this is also mediated by both $\sigma^E$ and $\sigma^S$ (Spector & Cubitt, 1992; Kenyon *et al.*, 2002). Both sigma factors also participate in the pathogenesis of *S. Typhimurium* infection (Fang *et al.*, 1992; Nickerson & Curtiss, 1997; Humphreys *et al.*, 1999; Testerman *et al.*, 2002; Rowley *et al.*, 2006).

Under conditions of starvation it is very important for bacterial cells to be able to regulate metabolic activity. $\sigma^E$ has been reported by Becker *et al.* (2005) to be involved in maintenance of a proton-motive force (PMF). These authors reported depolarization of the membrane potential (demonstrated by fluorescence ratio imaging) in a *S. Typhimurium* $rpoE$ mutant. Respiring cells are known to reduce a number of tetrazolium dyes and this has been employed to measure cellular viability (Roslev & King, 1993) and electron-transport system (ETS) activity (Blenkinsopp & Lock, 1990) in bacterial communities.

Although the regulons controlled by $\sigma^E$ and $\sigma^S$ are activated by several common stresses they also respond to distinct stimuli and their regulation is different (Hengge-Aronis, 2002; Alba & Gross, 2004; Rowley *et al.*, 2006). $\sigma^E$ is classified as an extracytoplasmic function sigma factor and primarily responds to envelope stress. In non-stress conditions, $\sigma^E$ responds to envelope stress. In non-stress conditions, $\sigma^E$ responds as an extracytoplasmic function sigma factor and primarily activates the proteolytic cascade is initiated by a motif present in the C terminus of certain outer-membrane proteins which interact with the PDZ domain of DegS (Walsh *et al.*, 2003). The regulation of $\sigma^S$ expression and activation is highly complex and occurs at the transcriptional, post-transcriptional, translational and post-translational levels (Hengge-Aronis, 2002).

In this study, we describe the contributions of both $\sigma^E$ and $\sigma^S$ in survival of *S. Typhimurium* at refrigeration temperature (4.5 °C) and in environments of different osmotic strength. We also investigated the effect of mutations in $rpoE$ and $rpoS$ on metabolic activity of *S. Typhimurium* during starvation.

**METHODS**

**Bacterial strains and culture conditions.** *S. Typhimurium* SL1344 wild-type (WT) (Hoisteth & Stocker, 1981) and mutant strains were stored at −80 °C on cryobeads (Prolab diagnostics). *S. Typhimurium* strains with null mutations in $rpoE$ (Humphreys *et al.*, 1999) or $rpoS$, and a double mutant ($rpoElrpoS$) (Kenyon *et al.*, 2002) were used. Prior to each experiment, the appropriate strain was recovered by spreading a bead onto Columbia agar containing 5 % defibrinated horse blood (BA; Oxoid) and incubated overnight at 37 °C. Luria–Bertani (LB) broth (Invitrogen) was used for routine liquid culture.

**Measurement of bacterial growth.** Growth was measured using a Bioscreen C automatic turbidometric analyser (Thermo Electron Corp.). Starter cultures were prepared by inoculating a single colony of the appropriate strain into LB followed by overnight incubation at 37 °C. This culture was diluted 1:100 into fresh, pre-warmed LB and 300 μl per well was transferred into a 100-well honeycomb Bioscreen plate. Growth was analysed at 37 °C with shaking every 2 min. To assess the effect of osmotic stress on growth, LB was supplemented with NaCl to a final concentration of 6 % (w/v).

**Starvation-survival assays.** Starter cultures were prepared by growing strains in 10 ml LB for 18 h at 37 °C. Starvation microcosms were prepared by inoculating the appropriate strain into 50 ml of either 0.85 % (saline) or 6 % (w/v) NaCl in 250 ml sterile flasks. The flasks were incubated statically in air at 4.5 or 37 °C. Each starter culture was diluted sequentially in saline in order to avoid the carry-over of any residual nutrients from the growth medium. Survival was determined by plating appropriate dilutions onto BA. Statistical significance was determined by one-way analysis of variance (ANOVA) for individual time points. The ANOVA calculated the probability ($P$) that survival of the mutant(s) differed from that of the WT. Due to the toxicity of 6 % (w/v) NaCl, cell death was more rapid, and we therefore used a higher starting inoculum to observe any differences in the pattern of survival at 37 °C.

**Measurement of metabolic activity.** As a measure of metabolic activity of the population, ETS activity was measured using a 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction assay, as previously described (Ozkanca & Flint, 1997). Briefly, to 1.0 ml of culture, 100 μl 0.2 % (w/v) INT (Sigma) was added followed by incubation for 2 h at either 4.5 or 37 °C. The reaction was stopped by adding 10 ml 37 % (v/v) formaldehyde and samples were centrifuged at 3000 g for 5 min. The supernatant was removed and the INT-formazan deposits were extracted by adding 1.0 ml methanol with incubation at 70 °C for 2 h. Finally, the samples were centrifuged at 13 800 g for 5 min and $A_{490}$ measured against a methanol-only control. Statistical analysis of ETS data was carried out using a two-tailed *t*-test.

**RESULTS AND DISCUSSION**

The *S. Typhimurium* strains were cultured in LB supplemented with NaCl to a final concentration of 6 % (w/v) as a model for growth during osmotic stress (Fig. 1). In LB + 6 % NaCl, the growth rate for all strains was reduced relative to growth in LB alone, and the $rpoE$, $rpoS$ and $rpoElrpoS$ mutants grew less rapidly than the WT (Fig. 1). The main effect of the increased osmolarity on the mutants compared to the WT strain was the extended lag phase of both the $rpoE$ and $rpoS$ mutants, which was particularly pronounced for the $rpoS$ mutant ( ~12 h). The duration of the lag phase of the $rpoElrpoS$ double mutant was even longer ( ~18 h),
which was approximately double that of the rpoE single mutant (~9 h) (Fig. 1). The growth defect of the rpoElrpoS double mutant was further exemplified by the finding that it grew more slowly in LB alone (Fig. 1). These findings indicate that both $\sigma^E$- and $\sigma^S$-regulated genes are required for optimal growth of S. Typhimurium in environments of high osmolarity, and this could contribute to the virulence defects of S. Typhimurium rpoE and rpoS mutants. The osmolarity of environments within the host is likely to be higher than that experienced by Salmonella spp. outside the host. Therefore, the S. Typhimurium rpoE and rpoS mutants may be less able to survive in such environments. Also, other regulators that are important for virulence in S. Typhimurium, such as OmpR, are activated by high osmolarity (Dorman et al., 1989). It is known that several $\sigma^E$-dependent genes play a role in the virulence of S. Typhimurium (Humphreys et al., 1999; Rowley et al., 2006). Therefore, high osmolarity may act as a general cue to inform pathogens such as S. Typhimurium that they are within a host. Activation of $\sigma^E$ and $\sigma^S$ in response to high osmolarity is likely to contribute to the virulence of S. Typhimurium by inducing the expression of genes required for survival in a variety of adverse conditions in the host, in addition to high osmolarity. The role of $\sigma^E$ in long-term survival under hyperosmotic conditions at refrigeration temperature has not been examined. We therefore went on to examine if $\sigma^E$ and $\sigma^S$ are involved in survival of S. Typhimurium at refrigeration temperature and 37°C in starvation-survival models.

In saline at 4.5°C, survival of the rpoE, rpoS and rpoElrpoS mutants was significantly ($P<0.05$) reduced relative to the WT strain. After an initial fall, the rpoE mutant survived almost as well as the WT parent over the first 48 days at 4.5°C (Fig. 2a). However, after this time, at day 60, the numbers of the rpoE mutant were significantly lower than the WT ($P<0.05$). Compared with the WT strain and the single mutants, duration of survival of the rpoElrpoS mutant was further reduced, and the number of viable cells was significantly ($P<0.05$) reduced at day 41 (Fig. 2a). Moreover, in 0.85% NaCl at 4.5°C, survival of the WT, rpoE, rpoS and rpoElrpoS strains became undetectable after 87, 60, 71 and 41 days, respectively (Fig. 2a). The survival defect of the rpoE mutant in saline was more severe at 37 than at 4.5°C (Fig. 2b). It is possible that incubation at the lower temperature results in the slower accumulation of aberrant proteins in the periplasm, which might contribute to this phenomenon. Survival of the rpoS mutant at 37°C was not significantly different to that of the WT strain over the first 7 days ($P>0.05$). However, after this time death of the rpoS mutant was significantly more rapid than for the WT strain ($P<0.05$) (Fig. 2b). In addition, at day 3, for example, rpoE and rpoElrpoS mutants survived significantly ($P<0.05$) less well than both WT and rpoS strains (Fig. 2b).

When the concentration of NaCl was increased to 6% ($\Delta w=0.967$) the contribution of $\sigma^S$ was more apparent. At both incubation temperatures survival of the rpoS and rpoE/
rpoS mutants was significantly ($P < 0.05$) shorter than that of the rpoE and WT strains (Fig. 2c, d). In addition, in 6% NaCl at 4.5°C, the duration of survival of the WT, rpoE, rpoS and rpoE/rpoS strains was 79, 64, 37 and 24 days, respectively (Fig. 2c). Thus, both sigma factors are required for full survival of S. Typhimurium under starvation conditions of high and low osmolarity at both 37 and 4.5°C. This further supports the concept of highly integrated regulatory networks in coordinating bacterial responses to stress where, in this case, there is probably some degree of overlap between σE- and σS-regulated genes (Fang, 2005).

To examine if inactivation of rpoE or rpoS affects ETS activity during starvation, ETS activity of cultures was measured in strains in stationary phase following growth in LB or starvation in saline at 37 and 4.5°C. ETS activity at 37°C of the rpoE, rpoS and double mutants was significantly ($P < 0.001$) higher than that of the WT parent after starvation in saline for 1 and 3 days (Fig. 3a). After 1 day starvation at 4.5°C (Fig. 3b), ETS activity levels were reduced 13-, 24-, 37- and 60-fold for WT, rpoE, rpoS and rpoE/rpoS strains, respectively, compared to starved cultures at 37°C. There was no statistical difference in the ETS activity for stationary-phase non-starved populations at either temperature ($P > 0.05$) (Fig. 3a, b). Elevated ETS levels of a σE-deficient population of S. Typhimurium have not been previously described. There are a number of possible explanations for this in the rpoE and rpoS mutants at 37°C. It is possible that a σE/σS-regulated gene down-regulates ETS activity. Alternatively, the inability to deal with the stress that the σE regulon is responsible for (such as accumulation of misfolded outer-membrane proteins) may overactivate other, energy-requiring, σE-independent stress-response systems. These observations are consistent with the finding that the σE regulon is involved in maintenance of a PMF in S. Typhimurium (Becker et al., 2005).

In the saline starvation-survival model used, nutrient starvation (Spector, 1998; Kenyon et al., 2002) and oxidative stress (Humphreys et al., 1999; Testerman et al., 2002; Kenyon et al., 2002) are likely to contribute to cell envelope stress and ultimately to death of the cell. The death rate of a bacterial population that has defects in regulation of metabolic activity under starvation conditions is thus likely to be more rapid. This was the case when the rpoE and rpoS mutants were starved, and may offer a physiological explanation for the reduced survival of these mutants at

![Fig. 2. Comparison of the ability of S. Typhimurium strains to survive in 0.85% NaCl at 4.5°C (a); 0.85% NaCl at 37°C (b); 6% NaCl at 4.5°C (c); and 6% NaCl at 37°C (d). The number of bacteria surviving was determined by viable count at time points post-inoculation. Each data point represents the mean of three experiments, and the error bar indicates the SEM. Enumeration was stopped on reaching the theoretical limit of detection, 3.33 c.f.u. ml⁻¹. In 6% NaCl at 37°C (d), a higher starting inoculum was used to observe any differences in the pattern of survival since preliminary experiments demonstrated more rapid loss of viability under these conditions, as described in Methods.](image-url)
other dehydrogenase genes in these mutants was decreased (Bang et al., 2005).

At 4.5 °C, metabolic activity of starved cells was reduced to very low levels (Fig. 3b). Reduction in the rate of energy-requiring processes at 4.5 °C is likely to contribute to the extended duration of survival compared to 37 °C. The finding that both $\sigma^S$ and $\sigma^E$ were important for viability in environments of differing osmotic strength, including at refrigeration temperature, suggests that there may be interaction between the $\sigma^E$ and $\sigma^S$ regulons, as has been shown for the response to oxidative stress in S. Typhimurium (Testerman et al., 2002; Bang et al., 2005). However, the fact that in all environments we examined the rpoE/rpoS double mutant grew or survived less well than the single rpoE or rpoS mutants indicates that the regulons do not completely overlap. Also, the importance of $\sigma^E$ and $\sigma^S$ to S. Typhimurium varies according to the environment.

In saline at 37 °C, the contribution of $\sigma^E$ was significantly greater over 7 days’ starvation than that of $\sigma^S$ (Fig. 2b). The importance of rpoE for survival at 4.5 °C suggests that, in S. Typhimurium, at least, some genes involved in prolonging survival at refrigeration temperatures are $\sigma^S$-regulated (Miticka et al., 2003; Rezuchova et al., 2003). Recently a large number of genes have been shown, or are suspected, to be $\sigma^S$-regulated in S. Typhimurium and E. coli. These include genes encoding proteins concerned with envelope homeostasis such as periplasmic proteases and folding factors but also many genes of unknown function that are predicted to encode inner- and outer-membrane proteins and genes that function in the cytoplasm (Rezuchova et al., 2003; Bang et al., 2005; Kabir et al., 2005; Rhodius et al., 2006; Skovierova et al., 2006). These genes can be targeted to determine which are important for coping with the stresses reported in this study. Interestingly, at least one of the genes identified, lpxP (dgg), which encodes a palmitoyl transferase that modifies lipid A, is known to be induced by cold-shock and therefore may be important for survival of S. Typhimurium at refrigeration temperatures during starvation (Skovierova et al., 2006).

$\sigma^S$ is important for both positive and negative regulation of starvation-inducible gene expression, such as the sti loci involved in phosphate, carbon and nitrogen starvation in Salmonella spp. and E. coli (O’Neal et al., 1994). The comparatively minor survival defects exhibited by the rpoS mutant over the initial stages of starvation in 0.85% NaCl (Fig. 2a, b) indicate that $\sigma^S$ and possibly other stress response regulators are more important than $\sigma^E$ for survival in this environment. Naturally occurring mutants in rpoS have been isolated from both clinical samples and the environment, where they may exhibit a fitness advantage. In one study, Salmonella rpoS mutants were found to grow more rapidly than wild-type strains in minimal medium containing propionate as the sole carbon source (Robbe-Saule et al., 2003). As many natural environments will be stress-inducing, we suggest there are likely to be few circumstances where loss of $\sigma^E$ function confers an obvious advantage.
survival advantage. It is interesting to speculate that extracytoplasmic stress response functions regulated by σE would be required for survival of Salmonella in other microenvironments where conditions of osmotic shock may be experienced (Mattick et al., 2000). These might include bile salts and foods with low αw properties, for example.

Conclusion

This study demonstrates that both σE- and σS-regulated genes are required for optimal growth of S. Typhimurium in media of high osmolarity and for long-term survival during starvation in simple solutions of different osmolarity at both refrigeration temperature and 37°C. In all cases the S. Typhimurium rpoE/rpoS double mutant exhibited the most severe phenotypic defects in terms of survival or growth. This indicates that both alternative sigma factors participate in maintaining bacterial viability in the different environments tested. However, the relative importance of σE and σS differed depending on the environment. In 6% NaCl, σS was more important than σE, whereas σE was more important than σS for survival in saline, especially at 37°C. Finally, these conditions are relevant to food preparation and storage and indicate that σE- and σS-regulated genes are required for optimal growth of S. Typhimurium in the food chain. It may also be expected that exposure of S. Typhimurium to conditions that activate the σE- or σS-pathways may enhance survival of the organism during food processing/storage.

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REFERENCES


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