The starvation-stress response of *Salmonella enterica* serovar Typhimurium requires σ^E , but not CpxR-regulated extracytoplasmic functions

William J. Kenyon,¹ D. Geary Sayers,¹ Sue Humphreys,² Mark Roberts² and Michael P. Spector¹

Author for correspondence: Michael P. Spector. Tel: +1 251 380 2688. Fax: +1 251 380 2711.
e-mail: mspector@usouthal.edu

¹ Department of Biomedical Sciences, University of South Alabama, Mobile, AL 36688, USA

² Department of Veterinary Pathology, Glasgow University Veterinary School, Glasgow G61 1QH, UK

Starvation of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) for an exogenous source of carbon and energy (C-starvation) induces the starvation-stress response (SSR). The SSR functions to (i) maintain viability during long-term C-starvation and (ii) generate cross-resistance to other environmental stresses. The SSR is, at least partially, under the control of the alternative sigma factor, σ^E . It is hypothesized that C-starvation causes cell envelope stresses that could induce the σ^E and/or Cpx regulons, both of which control extracytoplasmic functions and, thus, may play a role in the regulation of the SSR. In support of this hypothesis, Western blot analysis showed that the relative levels of σ^E increased during C-starvation, peaking after approximately 72 h of C-starvation; in contrast, CpxR levels remained relatively constant from exponential phase up to 72 h of C-starvation. To determine if σ^E , and thus the regulon it controls, is an essential component of the SSR, several mutant strains were compared for their abilities to survive long-term C-starvation and to develop C-starvation-induced (CSI) cross-resistances. An *rpoE* mutant strain was significantly impaired in both long-term C-starvation survival (LT-CSS) and in CSI cross-resistance to challenges with 20 mM H₂O₂ for 40 min, 55 °C for 16 min, pH 3.1 for 60 min and 870.2 USP U polymyxin B ml⁻¹ (PmB) for 60 min, to varying degrees. These results suggest that C-starvation can generate signals that induce the *rpoE* regulon and that one or more members of the σ^E regulon are required for maximal SSR function. Furthermore, evidence suggests that the σ^E and σ^S regulons function through separate mechanisms in the SSR. In contrast, C-starvation does not appear to generate signals required for Cpx regulon induction which support the findings that it is not required for LT-CSS or cross-resistance to H₂O₂, pH 3.1 or PmB challenges. However, it was required to achieve maximal cross-resistance to 55 °C. Therefore, σ^E is a key regulatory component of the SSR and represents an additional σ factor required for the SSR of *Salmonella*.

Keywords: *rpoE*, extracytoplasmic stress, sigma factors

INTRODUCTION

Serovars of *Salmonella enterica* encounter various microenvironments in the process of cycling through

Abbreviations: CRP, cAMP receptor protein; CSI, C-starvation-inducible/-induced; ECF, extracytoplasmic function; LT-CSS, long-term C-starvation survival; MS hiPCN, MOPS-buffered salts medium non-limiting in glucose, phosphate and nitrogen; MS loC, MOPS-buffered salts medium limiting for glucose (C-source); PmB, polymyxin B; *S. Typhimurium*, *Salmonella enterica* serovar Typhimurium; SSR, starvation-stress response.

their numerous host and non-host niches. Many of these environs are less than optimal for growth and can even threaten viability. Thus *Salmonella*, and related bacteria, must be capable of sensing and responding to such stresses to survive. Starvation for a carbon and energy source (C-starvation) is one of the most common stresses confronted by bacterial cells in environments outside of the laboratory (Foster & Spector, 1995; Spector, 1998). *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) and many other non-spore-forming bacteria

respond to C-starvation with major alterations in gene expression that result in morphologically and physiologically different and generally stress-resistant cells, when compared to growing cells. This is referred to as the starvation-stress response (SSR) (Spector & Cubitt, 1992; Foster & Spector, 1995; Spector, 1998). Unlike the stationary-phase response, the SSR is elicited (a) in response to a single specific parameter, carbon-energy source starvation, and (b) at cell densities 1 to 2 logs lower than those measured under typical stationary-phase conditions.

The SSR has two functions: (a) to combat the long-term effects of C-starvation and (b) to provide a general resistance to other environmental stresses such as extremes in temperature and pH, and exposure to H₂O₂ and antimicrobial peptides. A subset of SSR loci essential to long-term C-starvation survival (LT-CSS) and general stress resistance is under the control of the alternative σ factor encoded by the *rpoS* gene, σ^S (O'Neal *et al.*, 1994; Hengge-Aronis, 1996; Muffler *et al.*, 1997; Loewen *et al.*, 1998; Spector, 1998; Spector *et al.*, 1999). In addition, σ^S is essential for full oral virulence of *Salmonella* in the mouse virulence model system (Fang *et al.*, 1992; Loewen *et al.*, 1998; Ibanez-Ruiz *et al.*, 2000).

Out of our interest in studying the SSR of *Salmonella*, we became interested in further understanding the effects of C-starvation on extracytoplasmic functions (ECFs). Two pathways have been characterized in growing *Escherichia coli* cells that respond to extracytoplasmic stress and control ECF. One pathway is under the control of the alternative sigma factor, σ^E , encoded by the *rpoE* gene, and the other by the two-component sensor-regulatory system CpxRA. In *E. coli*, as well as *Salmonella*, σ^E is activated by stresses to the cell envelope and then directs the transcription of genes with ECFs (Erickson & Gross, 1989; Connolly *et al.*, 1997; Missiakas & Raina, 1998; Raivio & Silhavy, 1999). Unlike in *E. coli*, *Salmonella* strains lacking σ^E are not temperature-sensitive and σ^E is not an essential sigma factor during exponential-phase growth (Mecenas *et al.*, 1993; Hiratsu *et al.*, 1995; De Las Penas *et al.*, 1997a; Humphreys *et al.*, 1999). They are, however, sensitive to oxidizing agents and polymyxin B (PmB) when analysed using disk susceptibility tests on agar media (Humphreys *et al.*, 1999). Mutants of *rpoE* and at least one of the genes it is known to regulate in *E. coli*, *htrA* (also known as *degP*), exhibit significant attenuation in the murine salmonellosis model (Humphreys *et al.*, 1999). In *E. coli*, the *rpoE* regulon is believed to sense and respond to misfolded proteins in the outer membrane and/or periplasm, which can be caused by high temperature, ethanol and perhaps other stresses (Mecenas *et al.*, 1993; Hiratsu *et al.*, 1995; Raina *et al.*, 1995; Rouviere *et al.*, 1995; Missiakas *et al.*, 1997). In addition to the HtrA protease, members of the *E. coli* σ^E regulon include functions needed for envelope protein refolding (FkpA and SurA), *rpoE*/ σ^E regulation, virulence and other unidentified functions (De Las Penas *et al.*, 1997b; Danese & Silhavy, 1997; Missiakas *et al.*, 1997;

Missiakas & Raina, 1997a, b; Pallen & Wren, 1997; Betton *et al.*, 1998; Humphreys *et al.*, 1999; Spiess *et al.*, 1999; Nitta *et al.*, 2000).

In *E. coli*, the two-component regulator-sensor CpxRA system constitutes another extracytoplasmic stress response system (Pogliano *et al.*, 1997; Raivio & Silhavy, 1999). Some members of the CpxR regulon such as CpxP, HtrA and DsbC have been assigned anti-stress functions (Dartigalongue *et al.*, 2001; Danese & Silhavy, 1998; Skorko-Glonek *et al.*, 1999). Homologues of these genes have been identified in *Salmonella*. A major role attributed to the Cpx regulon is to assist in biosynthesis of P pili by dealing with misfolding or aggregation of subunits within the periplasm (Raivio & Silhavy, 1999).

Because C-starvation could result in cell envelope stresses and/or cause misfolding of cell envelope proteins that can activate the σ^E and/or Cpx regulatory pathways, we investigated the role of the alternative sigma factor σ^E and the two-component regulator-sensor CpxRA system in the SSR of *S. Typhimurium*. To determine if either or both of these regulons are required for the SSR, knock-out mutants of *rpoE* and *cpxR* were tested for their roles in the two known functions of the SSR, (a) LT-CSS and (b) generation of C-starvation-inducible (CSI) cross-resistance to other environmental stresses. The results reported here indicate that C-starvation generates signals capable of inducing the σ^E regulon, but not the CpxRA regulon, indicating that in addition to σ^S and cAMP-CRP (cAMP receptor protein), σ^E is a key regulator of the SSR of *S. Typhimurium*.

METHODS

Bacterial strains and transductions. The bacterial strains used in this study were all derivatives of the mouse-virulent strain of *S. Typhimurium*, SL1344, and are listed in Table 1. Desired mutations were transferred to appropriate strains via transduction with the high transducing derivative of P22 bacteriophage, P22 HT *int*-105 (Chan *et al.*, 1972). Transductants were determined to be non-lysogens by demonstrating sensitivity to the H5 derivative of P22 bacteriophage on Green Agar (Maloy, 1990).

Growth media and conditions used. The minimal media used in this study were modified MOPS-buffered salts (MS) media (Neidhardt *et al.*, 1974) containing either 0.4% (w/v) glucose (MS hiPCN) or 0.03% (w/v) glucose (MS loC) and are described in detail elsewhere (Spector & Cubitt, 1992; O'Neal *et al.*, 1994). Histidine was added to a final concentration of 0.2 mM. Ampicillin (Ap) and kanamycin (Km) were used at final concentrations of 30 and 50 $\mu\text{g ml}^{-1}$, respectively. Luria-Bertani (LB) agar (Difco) was used as a rich medium for viability plating.

LT-CSS assay. As described previously (Spector *et al.*, 1999), desired strains were grown overnight in MS hiPCN at 37 °C with aeration. Overnight cultures were diluted 1:100 into fresh MS hiPCN and grown to an OD₆₀₀ of 0.3–0.4 (exponential phase). One millilitre of this culture was then added to 9 ml MS medium lacking glucose (MS noC) in a 50 ml screw-cap flask. These cultures were then incubated at 37 °C with aeration for 21 d. At desired time points, aliquots of each

Table 1. Bacterial strains and plasmids used or constructed in this study

Strain or plasmid	Pertinent genotype (phenotype)*	Source or reference
Strains		
SL1344	<i>hisG46</i> (wild-type mouse-virulent <i>S. Typhimurium</i>)	Hoseith & Stocker (1981)
GVB311	SL1344 <i>rpoE</i> :: Ω -Km ^r	Humphreys <i>et al.</i> (1999)
GVB372	SL1344 <i>rpoE</i> :: Ω -Km ^r /pSH117	Humphreys <i>et al.</i> (1999)
GVB368	SL1344 <i>cpxR</i> :: Ω -Km ^r	This study
SMS438	SL1344 <i>rpoS</i> ::Ap ^r	O'Neal <i>et al.</i> (1994)
SMS592	SL1344 <i>rpoE</i> :: Ω -Km ^r <i>rpoS</i> ::Ap ^r	This study
Plasmids		
pWSK29	Ap ^r , low-copy-number vector	Wang & Kushner (1991)
pSH117	Wild-type <i>rpoE</i> cloned into pWSK29	Humphreys <i>et al.</i> (1999)
pTFP1	pRS1274::P1 _{<i>rpoE</i>} - <i>lacZ</i>	T. Testerman & F. Fang (unpublished)†
pTFP2	pRS1274::P2 _{<i>rpoE</i>} - <i>lacZ</i>	T. Testerman & F. Fang (unpublished)†

* Ap^r, Ampicillin resistance; Km^r, kanamycin resistance.

† University of Colorado Health Sciences Center, USA.

culture were serially diluted in MS buffer and plated in triplicate onto LB agar containing the necessary antibiotic(s) at 37 °C to determine viable counts. Survival was measured as the percentage of maximum viability achieved for the culture, typically ca. 5×10^8 c.f.u. ml⁻¹. Results are presented as mean values of at least three separate trials $\pm$ SEM.

Western blot analysis. Pellets of exponential-phase or C-starved cells were lysed with 0.1 % SDS and total protein was determined (Bio-Rad; Bradford, 1976). Proteins were separated by SDS-PAGE (Tris/HCl 4–15 % Ready Gels; Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). The membrane was incubated overnight at 4 °C with anti- σ^E antibody (polyclonal rabbit antibody) or anti-CpxR antibody (polyclonal rabbit antibody). The membrane was washed three times and then incubated at room temperature for 1 h in anti-rabbit HRP-conjugated antibody (Amersham Pharmacia Biotech). Following five washes, ECL reagents 1 and 2 (Amersham Pharmacia Biotech) were mixed 1:1 (v/v) and poured onto the surface of the membrane. After 1.5 min, the membrane was exposed to X-ray film (Kodak Biomax MR) and/or type 667 film (Polaroid) for various time intervals. The film was developed and the molecular mass of proteins was determined by reference to prestained standards (Gibco-BRL; very low range).

Assays for CSI cross-resistance to environmental stresses. Strains were grown overnight in MS hiPCN medium at 37 °C with aeration. Overnight cultures were then diluted 1:100 into either MS hiPCN to generate exponential-phase cells or into MS loC to generate C-starved cells. C-starvation was indicated by cessation of growth due to exhaustion of glucose from the MS loC cultures (final OD₆₀₀ of 0.3–0.4). Exponential-phase, 4–5 h C-starved and 24 h C-starved cells were challenged by diluting the culture 1:100 into one of the following challenge media: (a) MS buffer containing 20 mM H₂O₂, (b) NCE buffer (Neidhardt *et al.*, 1974) at pH 3.1, (c) MS buffer at 55 °C or (d) MS buffer containing 870.2 USP U PmB ml⁻¹. Viable counts were determined at various time intervals during each challenge as appropriate. Percentage survival was calculated by dividing the c.f.u. ml⁻¹ at the desired time point by the c.f.u. ml⁻¹ at time zero (100 % viability; typically ca. $3\text{--}5 \times 10^6$

c.f.u. ml⁻¹) and multiplying by 100. Results are presented as mean values of at least three separate trials $\pm$ SEM.

β -Galactosidase assay. Exponential-phase cells and 5, 24, 48 and 72 h C-starved cells carrying either pTFP1 or pTFP2 were assayed for β -galactosidase activity according to the method of Miller (1972).

RESULTS

σ^E , but not CpxR, is required for LT-CSS

One of the functions of the SSR is to allow *S. Typhimurium* to survive long periods of carbon-energy source starvation (Foster & Spector, 1995; Spector, 1998). It should be noted that we are starving cells at cell densities (approx. 10^8 c.f.u. ml⁻¹ or less) where cross-feeding from dead-lysed cells would be minimal (Spector & Cubitt, 1992). We hypothesized that long-term C-starvation could generate cell envelope or extracytoplasmic damage that must be dealt with to maintain cell viability. To test this hypothesis, we determined whether strains carrying knockout mutations in *rpoE* (encoding the alternative sigma factor, σ^E) or *cpxR* (encoding the regulator CpxR) exhibit LT-CSS comparable to the wild-type parent. Both σ^E and CpxR have been shown in *E. coli* to be major regulators of ECFs in response to extracytoplasmic stress (Erickson & Gross, 1989; Connolly *et al.*, 1997; Missiakas & Raina, 1998; Raivio & Silhavy, 1999).

As presented in Fig. 1, an *rpoE* knockout mutant, GVB311 (Humphreys *et al.*, 1999), showed a relatively rapid decline in culture viability and exhibited about 10-fold lower survival after 21 d of C-starvation, compared to its wild-type parent. In contrast, the *cpxR* knockout mutation did not appear to significantly impair LT-CSS, compared to the wild-type parent strain. This indicates that one or more members of the σ^E regulon, but not the CpxR regulon, plays an important role in LT-CSS.

Fig. 1. LT-CSS of *S. Typhimurium* wild-type, *rpoE* and *cpxR* mutant strains. Strains SL1344 (wild-type; ●; *n* = 8), GVB311 (*rpoE* mutant; ■; *n* = 8) and GVB368 (*cpxR* mutant; ▲; *n* = 7) were starved for a carbon and energy source for a total of 21 d as described in Methods. The data shown are mean values $\pm$ SEM from the indicated number (*n*) of separate experiments.

Western blot analysis shows that σ^E accumulates during C-starvation

The finding that an *rpoE* mutant was defective in LT-CSS prompted us to examine the relative levels of σ^E during long-term C-starvation. Western blot analysis of exponential-phase (LP) and C-starved wild-type cells of *S. Typhimurium* (SL1344) showed that the amount of σ^E protein increased relative to total cellular protein during C-starvation (Fig. 2a). In fact, σ^E levels continued to increase, peaking at about 48 h of C-starvation (Fig. 2a) and staying at that relative level for at least 144 h of C-starvation (data not shown). This relative accumulation of σ^E correlated well with the observed kinetics of the loss of viability of the *rpoE* mutant (Fig. 1), supporting a proposed role for one or more σ^E -regulated loci in coping with C-starvation.

σ^E -dependent transcription increases during C-starvation, indicating that all or part of the accumulated σ^E is active

Although Western blot analysis showed that σ^E protein accumulated during C-starvation, we wanted to determine if all or part of this protein was active (not sequestered by the anti-sigma factor RseA) in the starved

Fig. 2. Western blot analysis of σ^E levels and transcriptional activity of σ^E during C-starvation. Wild-type *S. Typhimurium* SL1344 was grown and treated as described in the Methods. (a) Ten micrograms of whole-cell protein was loaded per lane, electrophoresed, transferred to PVDF membrane and probed with antibody to σ^E . The blot shown is representative of at least three separate trials. (b) Wild-type *S. Typhimurium* possessing either pTFP1 (●) or pTFP2 (■) was grown to exponential phase (LP) and C-starved (C-st) for the time periods indicated. β -Galactosidase activity was measured by the procedure of Miller (1972). The data shown are mean values $\pm$ SEM from at least three separate experiments.

cell. For this, strains carrying the pTFP1 or pTFP2 plasmids were grown and C-starved for up to 144 h. pTFP1 contains the σ^E -independent P1 promoter of the *rpoE rseABC* operon fused to a promoterless *lacZ* gene, while pTFP2 contains the σ^E -dependent P2 promoter of the *rpoE rseABC* operon fused to a promoterless *lacZ* gene (T. Testerman & F. Fang, unpublished). In this way, we were able to monitor and compare the transcription from a σ^E -dependent and -independent promoter over the same time period that we observed σ^E accumulation.

Results, presented in Fig. 2(b), demonstrate that transcription from the σ^E -dependent promoter increased 4.5-fold during the first 5 h of C-starvation compared to exponential phase; this was followed by a further approximately 1.5-fold increase over the next 67 h of C-starvation. This, for the most part, mirrored the

Table 2. Effect of an *rpoE*, *cpxR* or *rpoS* mutation on CSI cross-resistance to various environmental stresses

Values shown are mean percentage survival $\pm$ SEM of the indicated number (*n*) of separate experiments. UD, c.f.u. ml⁻¹ levels were undetectable; ND, not done.

Pertinent genotype (<i>n</i>)	Exponential-phase cells	4–5 h C-starved cells	24 h C-starved cells
20 mM H₂O₂ for 40 min			
Wild-type (8)	0.20 $\pm$ 0.053	49.10 $\pm$ 7.31	59.70 $\pm$ 7.00
<i>rpoE</i> mutant (7)	UD	4.80 $\pm$ 4.78	10.70 $\pm$ 7.60
<i>rpoE</i> /pSH117 (3)	0.25 $\pm$ 0.10	47.30 $\pm$ 8.84	80.70 $\pm$ 1.90
<i>cpxR</i> mutant (5)	0.22 $\pm$ 0.19	43.80 $\pm$ 7.23	52.30 $\pm$ 5.20
<i>rpoS</i> mutant (3)	UD	UD	UD
<i>rpoE rpoS</i> mutant (4)	UD	UD	UD
55 °C for 16 min			
Wild-type (9)	UD	8.50 $\pm$ 3.06	13.80 $\pm$ 4.40
<i>rpoE</i> mutant (8)	UD	0.75 $\pm$ 0.57	0.38 $\pm$ 0.26
<i>rpoE</i> /pSH117 (3)	UD	3.80 $\pm$ 0.69	6.50 $\pm$ 0.75
<i>cpxR</i> mutant (4)	UD	1.10 $\pm$ 0.39	0.45 $\pm$ 0.13
<i>rpoS</i> mutant (3)	UD	UD	UD
<i>rpoE rpoS</i> mutant (3)	UD	UD	UD
pH 3.1 for 60 min			
Wild-type (10)	1.00 $\pm$ 0.41	52.80 $\pm$ 6.30	48.30 $\pm$ 4.76
<i>rpoE</i> mutant (6)	0.14 $\pm$ 0.061	2.40 $\pm$ 0.57	1.60 $\pm$ 1.07
<i>rpoE</i> /pSH117 (3)	4.70 $\pm$ 1.15	42.00 $\pm$ 1.15	80.00 $\pm$ 9.87
<i>cpxR</i> mutant (5)	1.70 $\pm$ 1.11	24.50 $\pm$ 4.49	32.80 $\pm$ 3.38
<i>rpoS</i> mutant (3)	0.07 $\pm$ 0.053	55.30 $\pm$ 9.91	0.042 $\pm$ 0.006
<i>rpoE rpoS</i> mutant (3)	UD	4.27 $\pm$ 1.95	0.015 $\pm$ 0.008
870.2 USP U PmB ml⁻¹ for 60 min			
Wild-type (13)	0.020 $\pm$ 0.005	49.20 $\pm$ 8.20	19.90 $\pm$ 6.75
<i>rpoE</i> mutant (8)	0.012	8.08 $\pm$ 1.77	0.61 $\pm$ 0.40
<i>rpoE</i> /pSH117 (9)	0.020	24.00 $\pm$ 0.35	6.20 $\pm$ 0.037
<i>cpxR</i> mutant (7)	0.020	34.00 $\pm$ 0.43	5.20 $\pm$ 0.32
<i>rpoS</i> mutant	ND	ND	ND
<i>rpoE rpoS</i> mutant	ND	ND	ND

increase in σ^E observed during Western blot analysis. In contrast, transcription from the σ^E -independent promoter remained relatively constant from exponential phase through 72 h of C-starvation. This indicates that a significant portion of the accumulated σ^E protein is active in C-starved cells.

These data also show that the *rpoE rseABC* operon is transcriptionally induced during C-starvation in a σ^E -dependent manner.

σ^E is needed for maximal CSI cross-resistance to H₂O₂, 55 °C, acid pH and the antimicrobial peptide PmB

Another consequence of induction of the SSR is a remarkable increase in the resistance of C-starved cells to a variety of other stresses (Hengge-Aronis, 1996; McLeod & Spector, 1996; Seymour *et al.*, 1996; Spector, 1998; Spector *et al.*, 1999). Therefore, we wanted to determine if the σ^E regulon, in addition to its role in LT-CSS, is important for the development of CSI cross-resistance to other environmental stresses.

Previous studies showed that cells must C-starve for 4–5 h to exhibit maximal CSI cross-resistance to most

other environmental stresses (Jenkins *et al.*, 1988; McLeod & Spector, 1996; Seymour *et al.*, 1996; Spector *et al.*, 1999). Therefore, desired strains were assayed for CSI cross-resistance to a (i) 40 min challenge with 20 mM H₂O₂, (ii) 16 min challenge at 55 °C, (iii) 60 min challenge at pH 3.1 and (iv) 60 min challenge with 870.2 USP U PmB ml⁻¹. Cells were challenged during exponential-phase growth and after 3–5 and 24 h of C-starvation.

As reported previously (Seymour *et al.*, 1996), survival of wild-type exponential-phase cells was undetectable, while 5 and 24 h C-starved wild-type cells exhibited 50–70 % survival after 40 min of H₂O₂ challenge (Table 2), a classic CSI cross-resistance phenotype. In comparison, survival of the *rpoE* mutant was impaired 10-fold in 5 h C-starved cells but only about 5- to 6-fold in 24 h C-starved cells. Complementation of the *rpoE* mutation through expression of σ^E from pSH117 in strain GVB372 (Humphreys *et al.*, 1999) restored CSI cross-resistance to wild-type levels (Table 2), indicating that the *rpoE* mutation is causing a real and reproducible effect. This indicates that one or more σ^E regulon members is essential for full CSI cross-resistance to

H₂O₂, although this appears to become less essential the longer the cells are C-starved (Table 2, 24 h C-starved *rpoE* mutant cells).

Thermotolerance has been demonstrated previously in C-starved cells (Jenkins *et al.*, 1988; Hengge-Aronis, 1996; Spector, 1998; Spector *et al.*, 1999). This, coupled with our findings that *rpoE* mutants are deficient in LT-CSS and CSI cross-resistance to H₂O₂, raised the question as to whether the σ^E regulon is required for CSI thermotolerance. Interestingly, previous studies found that *rpoE* mutants are temperature-sensitive in *E. coli*, but not in *Salmonella* (Mecenas *et al.*, 1993; Hiratsu *et al.*, 1995; De Las Penas *et al.*, 1997a; Humphreys *et al.*, 1999), and of the four known loci regulated by σ^E , three, *rpoH*, *htrA* and *fkpA*, are involved in thermotolerance in *E. coli* (Jenkins *et al.*, 1991; Mecenas *et al.*, 1993; Hiratsu *et al.*, 1995; Raina *et al.*, 1995; Rouviere *et al.*, 1995; Yura *et al.*, 1996; Missiakas & Raina, 1998; Raivio & Silhavy, 1999). Therefore, we investigated the role of σ^E in CSI cross-resistance to a 16 min challenge at 55 °C. As illustrated in Table 2, survival of C-starved *rpoE* mutants was reduced approximately 11- to 36-fold in both 5 and 24 h C-starved cells, respectively, when compared to the wild-type strain. Plasmid-borne σ^E again restored survival to wild-type levels in strain GVB372, indicating once more that these effects are real and reproducible (Table 2). Again, this demonstrates that one or more members of the σ^E regulon are needed for the generation of maximal protection against the damaging effects of thermal stress in C-starved cells.

C-starvation has also been shown to generate cross-resistance to acid pH (Foster & Spector, 1995; Spector, 1998; Spector *et al.*, 1999). Since we have already demonstrated a defective phenotype for an *rpoE* mutant in terms of various aspects of SSR function, we wanted to determine whether the σ^E regulon is required for CSI cross-resistance to a 40 min challenge at pH 3.1. In the case of wild-type cells, 5 and 24 h of C-starvation provided approximately 50-fold increased resistance to an external pH of 3.1, compared to exponential-phase cells (Table 2). Introduction of an *rpoE* knockout mutation reduced acid tolerance in 5 and 24 h C-starved cells by some 22- to 30-fold, respectively, compared to 5 and 24 h C-starved wild-type cells (Table 2). The finding that expression of σ^E from the *rpoE*⁺ containing plasmid pSH117 (GVB372) again restored cross-resistance to wild-type levels (Table 2) provides support for a role for the σ^E regulon in CSI cross-resistance to pH 3.1.

The cationic antimicrobial polypeptide PmB causes disruption of the outer membrane of Gram-negative bacteria (Daugelavicius *et al.*, 2000) and may introduce or cause extracytoplasmic damage. We have previously reported that C-starvation can generate cross-resistance to PmB (McLeod & Spector, 1996). Interestingly, unlike CSI cross-resistance to H₂O₂, 55 °C and pH 3.1, this form of cross-resistance is independent of the σ^S regulon and was found to develop after only 30 min of C-starvation, compared to 4–5 h for cross-resistance to other stresses, adding to the uniqueness of this form of CSI cross-resistance (McLeod & Spector, 1996). There-

Fig. 3. Western blot analysis of CpxR levels during C-starvation. Wild-type *S. Typhimurium* SL1344 was grown and treated as described in Methods. Three micrograms of whole-cell protein was loaded per lane, electrophoresed, transferred to PVDF membrane and probed with antibody to CpxR. The presented blot is representative of at least two separate trials.

fore, we wanted to determine if σ^E functions in CSI cross-resistance to a 60 min challenge with 870.2 USP U PmB ml⁻¹. As reported previously (McLeod & Spector, 1996), wild-type cells showed a substantial increase in resistance to a 60 min challenge with PmB after 3 and 24 h of C-starvation, respectively, when compared to exponential-phase cells. The *rpoE* mutant strain (GVB311) displayed a significant reduction in PmB resistance, particularly in 24 h C-starved cells, which was restored to wild-type levels by plasmid-borne expression of σ^E in strain GVB372 (Table 2). The *rpoE* mutant exhibited a reproducible 6-fold reduction in PmB resistance after 3 h of C-starvation and a more than 30-fold reduction in 24 h C-starved cells. Thus, one or more σ^E -regulated loci appear to be essential for maximal CSI cross-resistance to PmB, especially in 24 h C-starved cells. A role for the σ^E regulon in CSI cross-resistance to PmB suggests that at least part of the σ^E regulon functions through separate pathways from the σ^S regulon in the SSR, since the σ^S regulon plays no apparent role in CSI cross-resistance to PmB (McLeod & Spector, 1996).

Interestingly, exponential-phase *rpoE* mutant cells are hypersensitive to 20 mM H₂O₂, pH 3.1 and PmB challenges. This is similar to the effect that an *rpoS* mutation has on exponential-phase cells upon challenges with other environmental stresses (Table 2; Lange & Hengge-Aronis, 1991; McCann *et al.*, 1991; Seymour *et al.*, 1996; Foster & Spector, 1995; Spector *et al.*, 1999). Thus, like an *rpoS* mutant, an *rpoE* mutant is defective in all aspects of the SSR and hypersensitive to certain environmental stresses during exponential-phase growth.

CpxR is needed for CSI cross-resistance to 55 °C but not to H₂O₂, pH 3.1 or PmB

Although the *cpxR* mutation did not affect LT-CSS (Fig. 1), and according to Western blot analysis CpxR levels appear to decline slightly from exponential-phase levels and remain relatively constant during C-starvation (Fig. 3), we nonetheless wanted to examine the potential role for the Cpx regulon in CSI cross-resistance. Results presented in Table 2 indicate that the *cpxR* mutant was not significantly different from the wild-type parent in terms

Fig. 4. LT-CSS of *S. Typhimurium* wild-type, *rpoE*, *rpoS* and *rpoE rpoS* (double) mutant strains. Strains SL1344 (wild-type; ●; $n = 8$), GVB311 (*rpoE* mutant; ■; $n = 8$), SMS438 (*rpoS* mutant; ▲; $n = 1$) and SMS592 (*rpoE rpoS* double mutant, ◆; $n = 3$) were starved for a carbon and energy source for a total of 21 d as described in Methods. The data shown are mean values $\pm$ SEM from the indicated number (n) of separate experiments (except for the *rpoS* strain).

of CSI cross-resistance to a 40 min challenge with 20 mM H_2O_2 , a 60 min challenge at pH 3.1 or a 60 min challenge with 870.2 USP U PmB ml^{-1} .

Interestingly, the *cpxR* mutant was defective in CSI cross-resistance to a 16 min challenge at 55 °C. As illustrated in Table 2, the *cpxR* mutant exhibited similar reductions in percentage survival levels as the *rpoE* mutant, indicating that the Cpx regulon may play a role in thermotolerance generated in C-starved cells.

σ^E -regulated functions appear to act through different 'pathways' than σ^S -regulated functions in various aspects of the SSR in *Salmonella*

As previously shown in *E. coli* and *Salmonella* (reviewed by Foster & Spector, 1995; Hengge-Aronis, 1996; Spector, 1998), an *rpoS* knockout mutant exhibits deficiencies in both functions of the SSR, i.e. LT-CSS and CSI cross-resistance to other stresses, as well as showing hypersensitivity to environmental stresses in exponential phase. To get an idea of the interrelationship of the σ^S and σ^E regulons in the SSR, we performed similar LT-CSS assays on an *rpoS* mutant (SMS438) and

rpoE rpoS double knockout mutant (SMS592). As shown in Fig. 4, the *rpoS* mutant exhibited its typical 75- to 100-fold reduced level of survival after 21 d of C-starvation. The *rpoE rpoS* double mutant exhibited an even more rapid decrease in culture viability during C-starvation than the *rpoS* or *rpoE* mutant, ultimately displaying about 600-fold reduced survival after 21 d of C-starvation, compared to its wild-type parent. This suggests that the σ^S and σ^E regulons act through different 'pathways' or mechanisms to maintain cell viability during long-term C-starvation. However, it is possible that they may overlap in some components.

To characterize the interrelationship of the σ^E and σ^S regulons with respect to CSI cross-resistance, we subjected the *rpoS* mutant and *rpoE rpoS* double mutant to the various stress challenges after exponential-phase growth and 5 and 24 h of C-starvation. As reported previously (Jenkins *et al.*, 1988; Seymour *et al.*, 1996; Spector *et al.*, 1999) an *rpoS* mutant is severely deficient in CSI cross-resistance to H_2O_2 and heat challenge, exhibiting undetectable levels of viability after 40 min of H_2O_2 challenge or 16 min at 55 °C (Table 2). As a result of the drastic effect of the *rpoS* mutation alone, no additional effect could be discerned in the *rpoE rpoS* double mutant (Figs 3 and 4).

Interestingly, the *rpoE rpoS* mutant exhibited a comparable decrease in acid tolerance after 5 h of C-starvation, as did 5 h C-starved *rpoE* mutant cells. This is consistent with the finding that the *rpoS* mutant is not defective in CSI cross-resistance to pH 3.1 after 5 h of C-starvation (Table 2). However, in 24 h C-starved cells the affect of knocking out both σ^S and σ^E , in general, has a more dramatic effect than knocking-out each separately (Table 2), supporting the idea that the σ^S and σ^E regulons act through separate pathways or mechanisms in the SSR and the CSI cross-resistance to acid pH challenge.

It was reported previously (McLeod & Spector, 1996) that CSI cross-resistance to PmB challenge was σ^S -independent. So, we did not test an *rpoS* mutant or *rpoE rpoS* double mutant for CSI cross-resistance to PmB since such experiments would not yield additional useful information.

Additional findings that support the hypothesis that the σ^E and σ^S regulons appear to act through separate pathways has come from Western blot analysis of σ^E and σ^S accumulation in *rpoS* and *rpoE* mutants, respectively. σ^E accumulation during C-starvation was not significantly different in the *rpoS* mutant compared to the wild-type cell; similarly σ^S accumulation during C-starvation was not significantly different in the *rpoE* mutant compared to the wild-type strain (data not shown).

DISCUSSION

The results presented here indicate that C-starvation appears to generate signals capable of inducing the σ^E regulon and that one or more members of the σ^E regulon

are required for the two known functions of the SSR, LT-CSS and CSI cross-resistance to other environmental stresses in *S. Typhimurium*. A previous report (Humphreys *et al.*, 1999) suggested that *rpoE* mutants might be defective in the utilization of certain C-sources. This was inferred from the findings that an *rpoE* mutant, compared to wild-type strains, exhibited a longer lag phase and failed to reach as high an optical density after overnight growth in LB or M9 succinate medium in contrast to LB glucose or M9 glucose medium, respectively. This suggests that *rpoE* mutants might be deficient in the use of oxidatively metabolizable C-sources. Whether this deficiency is relevant to the reason why *rpoE* mutants survive long-term C-starvation poorly is unclear at this time.

The role of the σ^E regulon has been examined in stationary-phase cells where it has been proposed that σ^E controls the lysis of dead cells (Nitta *et al.*, 2000). However, these studies were performed on cultures at much higher cell densities and cells being subjected to multiple stress conditions simultaneously, e.g. pH stress, oxygen stress or excreted toxic metabolites, depending on the medium used. Nutrient starvation may or may not be the most important stress or the major limiting stress under these conditions. The studies described here were performed on cultures grown to much lower cell densities, up to 2 to 3 logs lower cell densities in some cases, and cells specifically subjected to starvation for an available exogenous carbon-energy source. For this reason it is unlikely that we are examining conditions similar to those described in Nitta *et al.* (2000). In that study the authors suggest that σ^E controls cell lysis and the amount of nutrients released from dead cells to maintain the viability of a subpopulation of viable cells. This could be hypothesized at the very high cell densities examined in that report where sufficient numbers of cells are dying and lysing to provide nutrients to support growth of other cells in the culture. However, at the cell densities examined here, such cross-feeding is unlikely to occur to any great extent (Spector & Cubitt, 1992).

The current model for the activation of the σ^E regulatory pathway in *E. coli* involves several steps involving up to three membrane and periplasmic proteins, encoded by *rseABC*, themselves part of the σ^E regulon (Connolly *et al.*, 1997; De Las Penas *et al.*, 1997b; Missiakas *et al.*, 1997; Missiakas & Raina, 1997a, 1998; Raivio & Silhavy, 1999). Basically in this model, the cytoplasmic domain of the membrane-bound anti-sigma factor RseA sequesters σ^E , making it unavailable to bind to RNA polymerase core enzyme. Extracytoplasmic stress leads to the release of σ^E from RseA by one or a combination of two possible mechanisms. In the first, RseB binds to the periplasmic domain of RseA and then dissociates from RseA when it 'senses' and binds misfolded proteins in the cell envelope. This causes a conformational change in RseA that results in σ^E release into the cytoplasm (Missiakas *et al.*, 1997; De Las Penas *et al.*, 1997b). In the second mechanism, a periplasmic protease, DegS, in response to misfolded proteins in the cell envelope, degrades RseA, causing the release of σ^E into

the cytoplasm (Ades *et al.*, 1999). In both cases, σ^E is made available for core RNA polymerase re-programming to increase transcription from σ^E -dependent promoters. This results in increased expression of σ^E itself as well as RseA and RseB. In addition to the *rpoE rseABC* operon, other reported members of the σ^E regulon in *E. coli* include *rpoH*, *htrA* (or *degP*), *fkpA* and other unidentified loci (Danese *et al.*, 1995; Connolly *et al.*, 1997; Danese & Silhavy, 1997; Missiakas & Raina, 1997a, 1998; Raivio & Silhavy, 1999).

Data presented in this paper indicate that (a) σ^E , but not CpxR, accumulates during C-starvation and (b) the σ^E regulon, but not the CpxR regulon, is required for LT-CSS (Fig. 1) and for CSI cross-resistances to other stresses in *S. Typhimurium* (Table 2). Therefore, we propose that C-starvation generates extracytoplasmic stress signals capable of activating the σ^E regulon, but not the CpxR regulon. If the mechanisms of activation described in *E. coli* are similar for *Salmonella*, then C-starvation generates extracytoplasmic signals that cause σ^E release from RseA, allowing for increased expression of σ^E and its regulon members (Fig. 2).

The finding that the σ^E regulon plays a key role in all aspects of the SSR tested to date suggests that C-starvation may lead to misfolding of or damage to proteins in the cell envelope, which would act as signals to induce the σ^E regulon. Many of the functions of known σ^E regulon members in *E. coli* involve repair of misfolded proteins such as the peptidyl prolyl isomerase (PPI) activities of FkpA and SurA (Dartigalongue *et al.*, 2001). Whether this is true for *Salmonella* is currently under investigation.

This study represents the first report indicating that the σ^E regulon, but not the Cpx regulon, is required for the maximal function of the SSR in *Salmonella*. The data presented here clearly show that σ^E plays a key role in regulating elements of the SSR of *S. Typhimurium*. As such, σ^E (along with σ^S and cAMP-CRP) functions as one of the master regulators of the SSR of *S. Typhimurium*. These data gives us some new insight into the types of stresses encountered by the bacterial cell at the molecular level during C-starvation. Furthermore, like σ^S and cAMP-CRP, σ^E has previously been found to be required for the full virulence of *S. Typhimurium* (Humphreys *et al.*, 1999). This further supports the hypothesis for a link between the SSR and virulence in this bacterium.

ACKNOWLEDGEMENTS

The authors would like to thank John Foster and Bruce White (University of South Alabama) for technical help and useful discussions. The authors are particularly grateful to Carol Gross and Susan Ades (University of California, San Francisco) for their kind gift of antibody to σ^E and Thomas Silhavy (Princeton University) for his kind gift of antibody to CpxR. The authors also wish to thank Traci Testerman and Ferric Fang (University of Colorado Health Science Center) for providing the pTFP1 and pTFP2 plasmids prior to publication. Portions of this work were supported by NIH grant no. AI/

OD-41170-01 and NSF grant no. MCB-9985981 (to M.P.S.) and BBSRC grants BFP11355 and PRS12222 (to M.R.)

REFERENCES

- Ades, S. E., Connolly, L. E., Alba, B. M. & Gross, C. A. (1999). The *Escherichia coli* σ^E -dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti- σ factor. *Genes Dev* 13, 2449–2461.
- Betton, J. M., Sassoon, N., Hofnung, M. & Laurent, M. (1998). Degradation versus aggregation of misfolded maltose-binding protein in the periplasm of *Escherichia coli*. *J Biol Chem* 273, 8897–8902.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72, 248–254.
- Chan, R. K., Botstein, D., Watanabe, T. & Ogata, Y. (1972). Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high-frequency-transducing lysate. *Virology* 50, 883–898.
- Connolly, L., De Las Penas, A., Alba, B. M. & Gross, C. A. (1997). The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. *Genes Dev* 11, 2012–2021.
- Danese, P. N. & Silhavy, T. J. (1997). The σ^E and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes Dev* 11, 1183–1193.
- Danese, P. N. & Silhavy, T. J. (1998). CpxP, a stress-combative member of the Cpx regulon. *J Bacteriol* 180, 831–839.
- Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. & Silhavy, T. J. (1995). The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev* 9, 387–398.
- Dartigalongue, C., Missiakas, D. & Raina, S. (2001). Characterization of the *Escherichia coli* σ^E regulon. *J Biol Chem* 276, 20866–20875.
- De Las Penas, A., Connolly, L. & Gross, C. A. (1997a). σ^E is an essential sigma factor in *Escherichia coli*. *J Bacteriol* 179, 6862–6864.
- De Las Penas, A., Connolly, L. & Gross, C. A. (1997b). The σ^E -mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E . *Mol Microbiol* 24, 373–385.
- Daugelavicius, R., Bakiene, E. & Bamford, D. H. (2000). Stages of polymyxin B interaction with the *Escherichia coli* cell envelope. *Antimicrob Agents Chemother* 44, 2969–2978.
- Erickson, J. W. & Gross, C. A. (1989). Identification of the σ^E subunit of *Escherichia coli* RNA polymerase, a second alternative σ factor involved in high-temperature gene expression. *Genes Dev* 3, 1462–1471.
- Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992). The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. *Proc Natl Acad Sci USA* 89, 11978–11982.
- Foster, J. W. & Spector, M. P. (1995). How *Salmonella* survive against the odds. *Annu Rev Microbiol* 49, 145–174.
- Hengge-Aronis, R. (1996). Regulation of gene expression during entry into stationary phase. In *Escherichia coli and Salmonella, Cellular and Molecular Biology*, 2nd edn, pp. 1497–1512. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Hiratsu, K., Amemura, M., Nashimoto, H., Shinagawa, H. & Makino, K. (1995). The *rpoE* gene of *Escherichia coli*, which encodes σ^E , is essential for bacterial growth at high temperature. *J Bacteriol* 177, 2918–2922.
- Hoseith, S. K. & Stocker, B. A. D. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 291, 238–239.
- Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A. B. & Roberts, M. (1999). The alternative sigma factor, σ^E , is critically important for the virulence of *Salmonella typhimurium*. *Infect Immun* 67, 1560–1568.
- Ibanez-Ruiz, M., Robbe-Saule, V., Hermant, D., Labrude, V. & Norel, F. (2000). Identification of RpoS (σ^S)-regulated genes in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 182, 5749–5756.
- Jenkins, D. E., Schultz, J. E. & Matin, A. (1988). Starvation-induced cross-protection against heat or H_2O_2 challenge in *Escherichia coli*. *J Bacteriol* 170, 3910–3914.
- Jenkins, D. E., Auger, E. A. & Matin, A. (1991). Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J Bacteriol* 173, 1992–1996.
- Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* 5, 49–59.
- Loewen, P. C., Hu, B., Strutinsky, J. & Sparling, R. (1998). Regulation in the *rpoS* regulon of *Escherichia coli*. *Can J Microbiol* 44, 707–717.
- McCann, M. P., Kidwell, J. P. & Matin, A. (1991). The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *J Bacteriol* 173, 4188–4194.
- McLeod, G. I. & Spector, M. P. (1996). Starvation- and stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in *Salmonella typhimurium* is RpoS (σ^S) independent and occurs through both *phoP*-dependent and -independent pathways. *J Bacteriol* 178, 3683–3688.
- Maloy, S. R. (1990). *Experimental Techniques in Bacterial Genetics*. Boston, MA: Jones & Bartlett.
- Mecas, J., Rouviere, P. E., Erickson, J. W., Donohue, T. J. & Gross, C. A. (1993). The activity of σ^E , an *Escherichia coli* heat-inducible σ -factor, is modulated by expression of outer membrane proteins. *Genes Dev* 7, 2618–2628.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Missiakas, D. & Raina, S. (1997a). Protein misfolding in the cell envelope of *Escherichia coli*, new signaling pathways. *Trends Biochem Sci* 22, 59–63.
- Missiakas, D. & Raina, S. (1997b). Signal transduction pathways in response to protein misfolding in the extracytoplasmic compartments of *E. coli*, role of two new phosphoprotein phosphatases PrpA and PrpB. *EMBO J* 16, 1670–1685.
- Missiakas, D. & Raina, S. (1998). The extracytoplasmic function sigma factors, role and regulation. *Mol Microbiol* 28, 1059–1066.
- Missiakas, D., Mayer, M. P., Lemaire, M., Georgopoulos, C. & Raina, S. (1997). Modulation of the *Escherichia coli* σ^E (RpoE) heat-shock transcription-factor activity by the RseA, RseB, and RseC proteins. *Mol Microbiol* 24, 355–371.
- Muffler, A., Barth, M., Marschall, C. & 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.

- Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.
- Nitta, T., Nagamitsu, H., Murata, M., Izu, H. & Yamada, M. (2000). Function of the σ^E regulon in dead-cell lysis in stationary-phase *Escherichia coli*. *J Bacteriol* **182**, 5231–5237.
- O'Neal, C. R., Gabriel, W. M., Turk, A. K., Libby, S. J., Fang, F. C. & Spector, M. P. (1994). RpoS is necessary for both positive and negative regulation of starvation-survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J Bacteriol* **176**, 4610–4616.
- Pallen, M. J. & Wren, B. W. (1997). The HtrA family of serine proteases. *Mol Microbiol* **26**, 209–221.
- Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C. C. & Beckwith, J. (1997). Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes Dev* **11**, 1169–1182.
- Raina, S., Missiakas, K. & Georgopoulos, C. (1995). The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. *EMBO J* **14**, 1043–1055.
- Raivio, T. L. & Silhavy, T. J. (1999). The σ^E and Cpx regulatory pathways, overlapping but distinct envelope stress responses. *Curr Opin Microbiol* **2**, 159–165.
- Rouviere, P. E., De Las Penas, A., Mecsas, J., Lu, C. Z., Rudd, K. E. & Gross, C. A. (1995). *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E , in *Escherichia coli*. *EMBO J* **14**, 1032–1042.
- Seymour, R. L., Mishra, P. V., Khan, M. A. & Spector, M. P. (1996). Essential roles of core starvation-stress response loci in carbon-starvation-inducible cross-resistance and hydrogen peroxide-inducible adaptive resistance to oxidative challenge in *Salmonella typhimurium*. *Mol Microbiol* **20**, 497–505.
- Skorko-Glonek, J., Zurawa, D., Kuczwara, E., Wozniak, M., Wypych, Z. & Lipinska, B. (1999). The *Escherichia coli* heat shock protease HtrA participates in defense against oxidative stress. *Mol Gen Genet* **262**, 342–350.
- Spector, M. P. (1998). The starvation-stress response (SSR) of *Salmonella*. *Adv Microb Physiol* **40**, 233–279.
- Spector, M. P. & Cubitt, C. L. (1992). Starvation-inducible loci of *Salmonella typhimurium*, regulation and roles in starvation survival. *Mol Microbiol* **6**, 1467–1476.
- Spector, M. P., Garcia del Portillo, F., Bearson, S. M., Mahmud, A., Magut, M., Finlay, B. B., Dougan, G., Foster, J. W. & Pallen, M. J. (1999). The *rpoS*-dependent starvation-stress response locus *stiA* encodes a nitrate reductase (*narZYWV*) required for carbon-starvation-inducible thermotolerance and acid tolerance in *Salmonella typhimurium*. *Microbiology* **145**, 3035–3045.
- Spiess, C., Beil, A. & Ehrmann, M. (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**, 339–347.
- Wang, R. F. & Kushner, S. R. (1991). Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195–199.
- Yura, T. & Nakahigashi, K. (1999). Regulation of the heat-shock response. *Curr Opin Microbiol* **2**, 153–158.
- Yura, T., Nakahigashi, K. & Kanemori, M. (1996). Transcriptional regulation of stress-inducible genes in procaryotes, In *Experientia Supplementum, Stress-Inducible Cellular Responses*, pp. 165–181. Edited by U. Feige and others. Basel: Birkhauser.

Received 19 July 2001; revised 24 September 2001; accepted 5 October 2001.