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Periplasmic peptidyl-prolyl isomerases SurA and FkpA play an important role in the starvation-stress response (SSR) of *Salmonella enterica* serovar Typhimurium

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Abstract

Carbon-energy source (C)-starved cells of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) are remarkably more resistant to stress than actively growing ones. Carbon-starved *S. Typhimurium* is capable of withstanding extended periods of starvation and assault from a number of different stresses that rapidly kill growing cells. These unique properties of the C-starved cell are the direct result of a series of genetic and physiological adaptations referred to as the starvation-stress response (SSR). Previous work established that the SSR of *S. Typhimurium* is partially regulated by the extracytoplasmic function sigma factor σ^E . As part of an effort to identify σ^E -regulated SSR genes, we investigated *surA* and *fkpA*, encoding two different classes of peptidyl-prolyl isomerase that function in folding cell envelope proteins. Both *surA* and *fkpA* are members of the heat-shock-inducible σ^E regulon of *Escherichia coli*. Although both genes are expressed in C-starved *Salmonella* cells, evidence indicates that *surA* and *fkpA* are not C-starvation-inducible. Furthermore, their expression during C-starvation does not appear to be σ^E -dependent. Nonetheless, *surA* and *fkpA* proved to be important, to differing degrees, for long-term C-starvation survival and for the cross-resistance of C-starved cells to high temperature, acidic pH, and the antimicrobial peptide polymyxin B, but neither were required for cross-resistance to oxidative stress. These results point to fundamental differences between heat-shock-inducible and C-starvation-inducible genes regulated by σ^E and suggest that genes other than *surA* and *fkpA* are involved in the σ^E -regulated branch of the SSR in *Salmonella*.

Keywords

Carbon-starvation; Cell envelope; Chaperone; Extracytoplasmic stress; Protein folding; Sigma factor

Introduction

The starvation-stress response (SSR) of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) represents a global readjustment in the pattern of gene expression and physiology of the bacterial cell (Foster and Spector 1995; Spector 1998). As *S. Typhimurium* depletes available sources of carbon and energy from the surrounding environment, the SSR is triggered and results in a physiologically distinct type of cell that is able to survive prolonged carbon-energy source starvation (C-starvation). In addition, C-starvation results in a cell that is remarkably resistant to other stresses encountered during its travels through various host and non-host environments. This aspect of the SSR is referred to as C-starvation-inducible (CSI) cross-resistance and includes increased tolerance toward high temperatures, extremes in pH, oxidizing conditions, and antimicrobial agents (Spector 1998). Suggesting an important overlap between the SSR and virulence, several SSR loci, including a few global regulatory factors, have proved to be critical for both maximal generation of the SSR and pathogenesis of *S. Typhimurium* in the mouse (Fang et al. 1992; O'Neal et al. 1994; Seymour et al. 1996; Humphreys et al. 1999; Spector et al. 1999).

Transition from exponential growth to C-starvation is essential for full induction of the SSR. By our experimental definition, C-starvation is a specific stress caused by exhaustion of glucose in minimal media at relatively low cell densities. Thus, C-starvation is different from stationary-phase (SP), a condition in which there are numerous stresses simultaneously imposed upon the bacterial cell. Many SSR genes are induced within the first 5-h of C-starvation and most of the remaining ones within 24-h following the onset of C-starvation (Spector and Cubitt 1992; Spector 1998; W. J. Kenyon and M. P. Spector, unpublished data).

Previous studies on the genetic regulation of the SSR have shown that the alternative sigma factor σ^S (σ^{38}), and the cAMP–CRP, complex are two master regulators responsible for induction of SSR genes in response to C-starvation (O'Neal et al. 1994; Spector 1998). We discovered that the SSR of *S. Typhimurium* is also under control of the *rpoE*-encoded alternative sigma factor σ^E (σ^{24}) (Kenyon et al. 2002). In *Escherichia coli*, σ^E regulates the expression of extracytoplasmic functions (ECF), many of which assist in the proper folding and assembly of cell envelope proteins (Mecas et al. 1993; Missiakas and Raina 1998; Raivio and Silhavy 2001; Ades 2008). In *S. Typhimurium*, σ^E levels rise in response to C-starvation and accumulation of σ^E causes an increase in σ^E -directed transcription (Kenyon et al. 2002). An *rpoE* null mutant of *S. Typhimurium* was significantly impaired in long-term C-starvation survival (LT-CSS) and in CSI cross-resistance to 55°C, pH 3.1, H₂O₂, and the antimicrobial peptide polymyxin B (PmB). In addition, Humphreys et al. (1999) showed that σ^E regulated genes are essential for survival and spread of *S. Typhimurium* in mice. We are now interested in identifying the individual genes involved in the σ^E -regulated branch of the SSR.

The finding that σ^E is activated in response to C-starvation suggested *S. Typhimurium* is experiencing some type of extracytoplasmic stress under these conditions (e.g., improper folding of cell envelope proteins). In *E. coli*, *surA* and *fkpA* were among the first group of genes to be identified as members of the σ^E regulon and the enzymes encoded by these genes combat cell envelope stress brought about by high temperatures (Dartigalongue et al. 2001). The periplasmic proteins SurA and FkpA represent two different classes of peptidyl-prolyl isomerases (PPIase), enzymes that catalyze the rate-limiting *cis-trans* isomerization of Xaa-Pro peptide bonds, and are important in the proper folding of proteins located in the cell envelope. SurA is a parvulin-like periplasmic PPIase involved in the proper folding and assembly of outer-membrane proteins (OMPs) (Lazar and Kolter 1996; Missiakas et al. 1996; Rouviere and Gross 1996; Behrens et al. 2001; Sklar et al. 2007). FkpA belongs to the FKBP family of proteins that bind the immunosuppressant drug FK506 (Horne and Young 1995; Ram and Pluckthun 2001; Arié et al. 2001). In addition to their PPIase activities, both

SurA and FkpA are capable of functioning as chaperones to prevent protein misfolding and aggregation in the cell envelope (Arié et al. 2001; Behrens et al. 2001).

In an earlier study, Humphreys et al. (2003) showed that *surA*, and to a lesser extent *fkpA*, are important for the survival of *S. Typhimurium* in the mouse. Inactivation of these genes also has an effect on the ability of *Salmonella* to invade and replicate within epithelial and macrophage cell lines (Humphreys et al. 2003).

We report here that *surA* and *fkpA* are expressed in both log-phase (LP) and C-starved (CS) cells of *Salmonella enterica* serovar Typhimurium. In addition, expression of these genes does not appear to be σ^E -dependent under the conditions tested, suggesting the presence of other σ^E -regulated SSR genes. However, we provide evidence that these periplasmic protein-folding factors have important roles to play in long-term C-starvation survival (LT-CSS) and in the ability of C-starved *Salmonella* cells to survive exposure to a wide range of environmental stresses.

Materials and methods

Bacterial strains and transductions

The two mouse-virulent *Salmonella enterica* serovar Typhimurium strains C5 and SL1344 were used as parental strains in this study. Strains derived from either C5 or SL1344 are listed in Table 1. Desired mutations were combined by transduction using the high transducing derivative of bacteriophage P22, P22 HT *int*-105 (Chan et al. 1972). Transductants were determined to be non-lysogens by demonstrating sensitivity to the H5 derivative of P22 on Green Agar (Maloy 1990).

Growth media and culture conditions

All cultures were grown aerobically at 37°C in modified MOPS-buffered salts (MS) minimal medium containing glucose as the sole carbon-energy source (Neidhardt et al. 1974; Spector and Cubitt 1992). MS hiC media contained an excess of glucose (0.4% w/v) and MS loC media contained a growth-limiting amount of glucose (0.03% w/v). LP cells were generated by growing cultures in MS hiC to an OD₆₀₀ of 0.3–0.4, and CS cells were generated by growing cultures in MS loC until growth ceased due to glucose depletion (at approximately the same OD₆₀₀ as MS hiC LP cultures). Luria–Bertani (LB) agar was used for viability plating. Ampicillin (Ap), kanamycin (Km) and chloramphenicol (Cm) were used when appropriate at final concentrations of 30, 50 and 45 µg ml⁻¹, respectively.

Complementation plasmids

Using oligonucleotide primers listed in Table 2 and purified *S. Typhimurium* C5 chromosomal DNA as template (Wizard Genomic DNA Purification Kit, Promega), the *surA* and *fkpA* ORFs along with their upstream promoter regions, were amplified using high-fidelity PCR (PCR SuperMix High Fidelity, Invitrogen) and cloned into the low-copy-number vector pWSK29 (Wang and Kushner 1991) using standard protocols (Sambrook et al. 1989) to generate plasmids pKS7 and pSH133, respectively. These plasmids, carrying a wild-type copy of each gene with potential promoter region, were then electroporated (BioRad *E. coli* Pulser Transformation Apparatus and BioRad protocol) into desired electrocompetent strains (Table 1) to test for complementation of mutant phenotypes.

Transcriptional *lacZ* fusions

Using primers listed in Table 2 and purified *S. Typhimurium* C5 chromosomal DNA as template (Wizard Genomic DNA Purification Kit, Promega), regions upstream *imp* (*ostA*), *surA*, *fkpA* and *rpoE* were amplified via high-fidelity PCR (PCR SuperMix High Fidelity) and

cloned by blunt ligation into the *Sma*I site preceding the promoter-less *lacZ* gene in pRS1274 (Simons et al. 1987) to generate plasmids pKS15, pKS18, pKS22 and pKS30, respectively. These plasmids were electroporated into both parental and *rpoE* null backgrounds (Table 1) in order to monitor transcriptional activity in log-phase and C-starved cells. β -Galactosidase activity was measured using the method of Miller (1992). β -Galactosidase activity is expressed in Miller units and presented as the mean $\pm$ the standard error from the mean (SEM) from at least three separate experiments.

RNA slot-blot analysis

Total RNA was extracted and purified from LP and CS cells using RNAwiz (Ambion). Contaminating DNA was removed by treatment with DNase I (Ambion DNA-free kit). Total RNA was quantitated at A₂₆₀ and stored at -80°C . Equal quantities of total RNA from LP, 5-h CS and 24-h CS cells were loaded into separate slots of a Bio-Slot SF (BioRad) apparatus and transferred to a Zeta-Probe GT nitrocellulose membrane (BioRad) under vacuum. Blots were then processed according to the Northern hybridization protocol described by Kenyon et al. (2007). Chemiluminescent detection of mRNA was accomplished using a PCR-generated Digoxigenin (DIG)-labelled probe (PCR DIG Probe Synthesis Kit; Roche Applied Science) as described by Kenyon et al. (2007). Images were obtained using an ECL mini-camera (Amersham Pharmacia Biotech).

Western blotting

Western blots were performed as described previously (Kenyon et al. 2002) using polyclonal antibody raised against SurA (anti-SurA was provided by Susanne Behrens, Georg-August-Universität Göttingen).

Long-term C-starvation survival

As described elsewhere (Spector et al. 1999; Kenyon et al. 2002), LT-CSS was monitored by diluting 1 ml of a mid-log-phase ($\text{OD}_{600} = 0.3\text{--}0.4$) MS hiC culture in 9 ml of fresh MS noC (no glucose) media and incubating at 37°C with shaking for up to 21 days. Viability counts were ascertained at pre-determined intervals by removing an aliquot, performing 10-fold serial dilutions in MS buffer and plating each dilution in triplicate on LB agar containing appropriate antibiotics. Survival is reported as a percentage of the maximum viability reached for that culture, and data is presented as the mean $\pm$ the standard error from the mean (SEM) from at least three separate experiments.

Cross-resistance challenge assays

Assays for cross-resistance were performed as described previously (Kenyon et al. 2002). In brief, strains were grown in either MS hiC media to generate LP cells, or in MS loC media to generate 5-h and 24-h CS cells, and diluted 1:100 into one of the following challenge buffers: MS buffer at 55°C , NCE buffer (Neidhardt et al. 1974) at pH 3.1, MS buffer containing 15 mM H_2O_2 or MS buffer containing 870.2 USP units ml^{-1} of polymyxin B (PmB). Viability counts were performed at pre-determined time intervals as described above. Survival is reported as a percentage of that at time zero (100%; typically $\sim 5 \times 10^6$ c.f.u. ml^{-1}) and presented as the mean $\pm$ SEM from at least three separate experiments.

Results

Promoter-*lac* transcriptional fusions

Initial studies established σ^E as one of the master regulators of the SSR. Subsequently, we have begun to investigate several genes as potential members of the σ^E -regulated branch of the SSR. The genes *surA* and *fkpA* are logical candidates because these genes are known to be regulated

by σ^E in both *E. coli* and *S. Typhimurium* (Dartigalongue et al. 2001; Rhodius et al. 2006; Skovierova et al. 2006). In both organisms, *surA* is the second gene of a large operon (Tormo et al. 1990; Pease et al. 2002; Skovierova et al. 2006). In *E. coli*, a σ^E promoter lies upstream of the first gene in the operon known as *ostA* (*lptD*), or *imp* in *Salmonella*, and is believed to control expression of the downstream *surA* gene (Dartigalongue et al. 2001). The corresponding region in *S. Typhimurium* differs at the -10 site and in the spacer region between the -10 and -35 sites (Dartigalongue et al. 2001; Skovierova et al. 2006). A second σ^E promoter thought to control *surA* expression has been identified in *S. Typhimurium* and is located within the *imp* ORF (Skovierova et al. 2006). Surprisingly, transcription of *surA* is σ^E -independent in SP cultures of *S. Typhimurium* (Bang et al. 2005; Skovierova et al. 2006). Furthermore, RNA slot-blot (Fig. 1), Western blot (Fig. 2) and microarray analyses (M. Antonio, S. Lucchini, R. Chaudhuri, A. Thompson, J. Hinton, M. P. Spector and M. J. Pallen, unpublished data) all suggest that *surA* is expressed in LP and CS cells but is not a C-starvation-inducible (CSI) gene. We provide evidence below that a third promoter, located somewhere within the intergenic region between *imp* and *surA*, may contribute to the expression of *surA* in CS cells of *S. Typhimurium*. In contrast, the *fkpA* σ^E promoter sequences of *S. Typhimurium* and *E. coli* are 100% identical (Skovierova et al. 2006).

S. Typhimurium chromosomal sequences upstream of *imp*, between *imp* and *surA*, and upstream of *fkpA* were PCR amplified with a high-fidelity polymerase and fused to the promoter-less *lacZ* gene present in pRS1274 (Simons et al. 1987; Table 3). None of these transcriptional fusions proved to be CSI during the time period when an *rpoE* null mutant was shown to be impaired in various aspects of the SSR (Kenyon et al. 2002; Table 3), but all promoter-*lac* fusions were transcriptionally active in LP and CS cells. Interestingly, these data demonstrate a relatively low level of *surA* expression from a promoter located between *imp* and *surA*. In fact, the *imp-surA* intergenic region does appear to harbor at least two potential σ^{70} promoter sequences. A transcriptional start site (TSS) is predicted precisely 10 bp upstream of the *surA* start codon (NNPP Prokaryotic Promoter Prediction, BCM Search Launcher, Baylor College of Medicine Human Genome Sequencing Center). Further analysis revealed a potential σ^{70} promoter with a -10 site starting 6 bp upstream of the predicted TSS (GTTAAT; 4 of 6 matches to σ^{70} consensus) and a -35 site starting 29 bp upstream of the predicted TSS (TTTACA; 5 of 6 matches), with a 17 bp spacer sequence. A second potential σ^{70} promoter was also identified with a -10 site starting 22 bp upstream of the predicted TSS (CGTAAT; 4 of 6 matches to σ^{70} consensus) and a -35 site starting 44 bp upstream of the predicted TSS (ATGTAA; 3 of 6 matches), with a 16 bp spacer sequence. No sequences resembling other consensus sigma factor binding sites were found in the intergenic region. Both potential σ^{70} promoters show some divergence from the σ^{70} consensus at key nucleotides, and this may account for the low level of expression observed from the *surAP::lac* fusion relative to the *impP::lac* and *fkpAP::lac* fusions (Table 3). For comparison, we included a fusion of the entire region upstream of the *rpoE rseABC* operon with the *lac* reporter (*rpoEP::lac*) in pRS1274. This transcriptional fusion shows an approximately 1.4-fold increase in activity over log-phase levels by 24-h of C-starvation (Table 3). This induction is not observed in an *rpoE* null background and is consistent with our previous findings using plasmids pTFP1 and pTFP2 carrying the individual fusions *rpoEP1::lac* (σ^{70} -dependent) and *rpoEP2::lac* (σ^E -dependent), respectively (Kenyon et al. 2002). The level of transcription driven by the *surAP::lac* and *fkpAP::lac* fusions actually increases in an *rpoE* null background (Table 3), indicating expression of these genes does not depend upon σ^E under these culture conditions. Sigma factor competition for RNA polymerase core complex is a possible explanation for the overall increase in expression we observed in an *rpoE* background. In the absence of σ^E , more RNA polymerase core complex is likely to become available for binding to σ^{70} , thus leading to an increase in expression from σ^{70} promoters.

Slot-blot analysis of *surA* mRNA levels

Slot-blot analysis was performed on total RNA isolated from LP and CS cells, and a DIG-labelled probe was used to specifically detect mRNA containing the *surA* sequence via chemiluminescence. Results shown in Fig. 1 reveal a notable decline in mRNA transcripts originating from all possible *surA* promoters after 5-h of C-starvation and a further decline after 24-h of C-starvation in both the parental and *rpoE* null backgrounds. The overall level of *surA* containing transcripts appears to be higher in the *rpoE* null background compared to the parental background, particularly in 24-h CS cells. This supports our transcriptional fusion data for the *impP::lac* and *surAP::lac* fusions in both parental and *rpoE* null backgrounds (Table 3). No chemiluminescent signal was detected in a Δ *surA* background (data not shown). Therefore, the level of *surA*-containing transcripts does not increase in response to C-starvation. Furthermore, the presence of *surA* mRNA in both the parental and *rpoE* backgrounds, further indicates that transcription of *surA* is σ^E -independent under these culture conditions.

Western blot analysis of SurA protein levels

Western blotting was used to detect changes in SurA protein levels during the transition from LP growth to CS in minimal media. Our results show no increase in SurA protein at 5- and 24-h of C-starvation relative to LP levels (Fig. 2). In addition, the level of SurA remained unchanged in the *rpoE* null background. Figure 2 also illustrates that SurA protein levels do not increase in stationary-phase (SP) cells compared to LP cells and that SurA levels are not dependent on σ^E in SP cells. These observations from cultures grown in MS minimal media are in agreement with previous reports of SP cells grown in LB medium (Bang et al. 2005; Skovierova et al. 2006).

Thus, the transcriptional fusion data, RNA slot-blot results and Western blot results all lead to the conclusion that *surA* is not induced during the first 24-h of C-starvation relative to exponentially growing cells. In fact, overall transcription of *surA* appears to decline in CS cells while SurA protein levels remain comparatively constant. These data also show that expression of both *surA* and *fkpA* is σ^E -independent during the time frame in which σ^E is required for a maximal SSR (Kenyon et al. 2002). These results suggest fundamental differences between the heat-shock-inducible σ^E regulon of *E. coli* and the C-starvation-inducible σ^E regulon of *S. Typhimurium*.

Long-term C-starvation survival

The physiological changes that occur during the SSR enable salmonellae to withstand prolonged periods of C-starvation. Our previous studies with an *rpoE* null mutant demonstrated that the absence of σ^E results in an approximate 10-fold drop in long-term C-starvation survival (LT-CSS) compared to the parental *S. Typhimurium* strain (Kenyon et al. 2002). Although our data indicate that expression of *surA* and *fkpA* is not inducible or σ^E -dependent during C-starvation, both genes are expressed in CS cells. Therefore, we wanted to know if inactivation of *surA* and/or *fkpA* would have an effect on LT-CSS. Using the *S. Typhimurium* Δ *surA* strain constructed by Sydenham et al. (2000), we found that loss of *surA* had a significant impact on LT-CSS (Fig. 3). The deletion in this strain does not exert a polar effect on expression of genes located downstream of *surA* (Sydenham et al. 2000). Similarly, Tormo et al. (1990) found that a mutation in *surA* of *E. coli* significantly affected survival during stationary-phase in LB medium. Interestingly, Lazar et al. (1998) reported that this *surA* phenotype of *E. coli* is due to a σ^S -related defect in the ability of stationary-phase cells to begin growing again after a pH rise in peptide-rich media such as LB. In contrast, we used a minimal medium strongly buffered with MOPS (Spector and Cubitt 1992) and an *rpoS*⁺ strain of *S. Typhimurium* (Sydenham et al. 2000). Therefore, we do not believe we are observing the same phenotype, but it is possible that SurA is needed for the recovery of cells on LB agar following C-starvation. Disruption of the *fkpA* gene alone had no effect on LT-CSS, but when combined with the *surA* deletion,

survival dropped below that of the *surA* single mutant, and was even less than that of an *rpoE* mutant, after 21 days of C-starvation (Kenyon et al. 2002; Fig. 3). These results suggest that SurA can functionally compensate for the absence of FkpA, but when SurA is missing, or especially when both SurA and FkpA are missing, the ability of cells to survive or recover from prolonged periods of C-starvation is significantly impaired. It is also possible that other periplasmic protein-folding factors could functionally compensate for the absence of SurA and/or FkpA. In fact, *S. Typhimurium* possesses at least two other periplasmic PPIases, PpiA (RotA) (Pogliano et al. 1997) and PpiD (Dartigalongue and Raina 1998). However, expression of these two enzymes is controlled by the CpxRA extracytoplasmic stress response which was previously shown not to contribute to the SSR (Kenyon et al. 2002), although this does not entirely rule out possible involvement of PpiA or PpiD under our test conditions.

CSI cross-resistance to heat challenge

Compared to actively growing cells, C-starved cells of *S. Typhimurium* display an increased tolerance towards a wide spectrum of potentially lethal stresses. This aspect of the SSR is referred to as C-starvation-induced (CSI) cross-resistance. Our previous studies had shown that *rpoE* is essential for the development of full CSI cross-resistance to high temperature (55°C), acidic pH (pH 3.1), oxidation (H₂O₂), and the antimicrobial peptide polymyxin B (PmB) (Kenyon et al. 2002). Although expression of *surA* and *fkpA* proved not to be CSI or σ^E -dependent, we discovered that these periplasmic factors do contribute to some forms of cross-resistance in CS cells.

Table 4 presents the survival data obtained from subjecting actively growing LP, 5-h CS and 24-h CS cells of *S. Typhimurium* to thermal challenge (55°C for 16 min). Deletion of the *surA* gene caused an 11.8-fold and 7.4-fold drop in thermotolerance in 5-h and 24-h of CS cells, respectively, relative to the parental strain ST251. Confirming that this phenotype was due to the loss of *surA*, survival was restored to near parental levels by supplying a wild-type copy of the *surA* gene (*imp-surA* intergenic region plus the *surA* ORF) on a low-copy-number vector (plasmid pKS7). The presence of an internal *surA* promoter between *imp* (*ostA*) and *surA* in *S. Typhimurium* was implied by the work of Tamayo et al. (2002). The fact that pKS7 plasmid complements *surA* SSR phenotypes, along with our transcriptional fusion data indicating promoter activity in this intergenic region (Table 3), strongly supports the existence of such a promoter.

Disruption of the *fkpA* gene resulted in a 47.9-fold and 32.1-fold decrease in thermotolerance in 5-h and 24-h CS cells, respectively, compared to the parental strain. Plasmid pSH133, carrying a wild-type copy of *fkpA*, complemented the *fkpA* phenotype. The level of survival of a *surA fkpA* double mutant was approximately the same as that of the *fkpA* single mutant, suggesting that FkpA plays a more important role than SurA in preventing or repairing the damage caused by thermal stress in CS cells.

CSI cross-resistance to acid pH challenge

Table 4 also presents the survival data obtained from subjecting actively growing LP, 5-h CS and 24-h CS cells of *S. Typhimurium* to acidic pH challenge (pH 3.1 for 40 min). Interestingly, the loss of either *surA* or *fkpA* greatly diminished the acid tolerance (pH 3.1 for 40 min) of 24-h CS cells (25.6- and 79.1-fold, respectively) whereas neither mutation had much affect on the acid tolerance of 5-h CS cells (Table 4). Plasmids pKS7 and pSH133 were again able to restore survival to levels comparable to the parent ST251. The *surA fkpA* double mutant showed an even greater decline in acid tolerance in 24-h CS cells. In contrast to the single mutants, 5-h CS double mutant cells showed a 10-fold decline in acid tolerance compared to the parental strain. These findings again suggest that SurA and FkpA may be able to compensate for each

other but also indicate that other factors are responsible for protecting *S. Typhimurium* against the harmful effects of low pH, especially during the first few hours of C-starvation.

CSI cross-resistance to hydrogen peroxide challenge

In contrast to *rpoE*, *surA* and *fkpA* appear to be dispensable for the cross-resistance of CS *Salmonella* cells to oxidative stress (15 mM H₂O₂ for 40 min) as shown in Table 4. Although there was a slight reduction in the survival of the *surA* and *fkpA* mutants relative to the parental strain (approximately 50% and 25%, respectively), there was virtually no difference in the cross-resistance exhibited by the parent and the *surA fkpA* double mutant.

CSI cross-resistance to polymyxin B challenge

Carbon-starved cells were previously found to exhibit cross-resistance to a 60 min challenge with the antimicrobial peptide polymyxin B (McLeod and Spector 1996). Thus, we wanted to determine if FkpA and/or SurA might play a role in this cross-resistance as well. Inactivation of *fkpA* had no effect on cross-resistance to polymyxin B (PmB; 60 min exposure to 870.2 USP units ml⁻¹), whereas deletion of *surA* caused a dramatic increase in the susceptibility of both LP and CS cells to PmB (data not shown). Consequently, PmB challenges were impossible to perform using strains carrying the *surA* mutation. This confirms a similar observation made by Tamayo et al. (2002) who reported that a MudJ insertion 23 bp from the end of *imp* (*ostA*) in *S. Typhimurium* greatly affected PmB resistance because of a polar effect on *surA* expression. Polymyxin B disrupts the outer-membrane (initial event) and inner-membrane (lethal event) of Gram-negative bacteria, thus affecting the permeability of these important barriers to the external environment (Daugelavicius et al. 2000; Pálffy et al. 2009). Therefore, it is conceivable that SurA, known to be involved in the folding of OMPs (Lazar and Kolter 1996; Sklar et al. 2007), may function to repair, or prevent damage to, the outer-membrane upon exposure to PmB and may consequently limit damage to the inner-membrane.

Discussion

Salmonella enterica serovars must cope with a variety of stressful microenvironments that can inhibit their growth or even threaten viability. One common stress encountered by salmonellae is the lack of an exogenous carbon-energy source (C-starvation). The changes in cellular gene expression and physiology that occur during transition from non-limiting exponential growth to C-starvation conditions enable the cell to withstand the effects of continued C-starvation as well as exposure to other stresses that often accompany C-starvation such as high temperature, low pH, oxidizing environments and biological compounds with antimicrobial activity. We refer to these changes collectively as the starvation-stress response (SSR) (Foster and Spector 1995; Spector 1998).

In both *E. coli* and *S. Typhimurium*, the alternative sigma factor σ^E (σ^{24}), encoded by the *rpoE* gene, controls the expression of genes that respond to cell envelope (extracytoplasmic) stress, and many other bacteria possess σ^E homologs (Missiakas and Raina 1997; Missiakas and Raina 1998; Raivio and Silhavy 1999; Raivio and Silhavy 2001; Rhodius et al. 2006; Rowley et al. 2006; Ades 2008). Because σ^S (σ^{38}), encoded by the *rpoS* gene, regulates the expression of several SSR genes whose products function primarily in the cytoplasm, we hypothesized that σ^E might be responsible for regulating genes that cope with extracytoplasmic stress associated with C-starvation. Using an *rpoE* null mutant, we previously found that σ^E is critical for LT-CSS and CSI cross-resistance to other stresses, and we also presented evidence that σ^E accumulates during C-starvation at the same time that σ^E -dependent transcription increases (Kenyon et al. 2002). These findings proved that σ^E is a second master regulator of the SSR in *S. Typhimurium*. An *rpoS rpoE* double mutant proved to have an even more severe SSR phenotype than either single mutant (Kenyon et al. 2002). In fact, it was apparent from

LT-CSS experiments that the effect was additive, suggesting minimal overlap between the SSR functions regulated by σ^E and σ^S . This work established that regulation of the SSR is under the control of at least two alternative sigma factors and that these sigma factors likely control separate SSR pathways.

In both *E. coli* and *Salmonella*, the classical *rpoH*-encoded, heat-shock sigma factor σ^H (σ^{32}) was the first *E. coli* σ^E regulon member to be identified (Erickson and Gross 1989; Wang and Kaguni 1989). This discovery was largely the result of research on the heat-shock-inducible *rpoHP3* promoter. Since that time, there have been many more σ^E -regulated genes identified in *E. coli* and *S. Typhimurium*, and there is now a canonical RNA polymerase- σ^E binding site sequence (Dartigalongue et al. 2001; Rezuchova et al. 2003; Rowley et al. 2006; Skovierova et al. 2006). To begin identifying σ^E -regulated SSR genes, we wanted to investigate the possible involvements of *surA* and *fkpA*. These genes are members of the heat-shock-inducible σ^E regulon of *E. coli*, and known or putative σ^E promoters are located upstream of both genes in *E. coli* and *S. Typhimurium* (Dartigalongue et al. 2001; Skovierova et al. 2006). Using transcriptional fusions as well as RNA slot-blot and Western blot analyses, we have found that neither *surA* nor *fkpA* is up-regulated in 5-h or 24-h CS cells of *S. Typhimurium* when compared to LP cells. Furthermore, expression of these two genes is unaffected by the σ^E status of the cell during exponential-phase growth or C-starvation. Others have previously reported that transcription of *surA*, *fkpA* and the *degP* ortholog *htrA* is σ^E -independent in LB stationary-phase cultures of *S. Typhimurium* (Bang et al. 2005; Lewis et al. 2009). Additionally, Lazar et al. (1998) showed that SurA protein levels were practically identical during LP and SP growth of *E. coli* in LB medium. Similarly, we have observed constitutive σ^E -independent expression of HtrA (DegP) in LP and CS cells of *S. Typhimurium* (W. J. Kenyon and M. P. Spector, unpublished data). Intriguingly, a comparison of results from Skovierova et al. (2006) and data from microarray analysis (M. Antonio et al., unpublished data) suggest that only some members of the *S. Typhimurium* σ^E regulon appear to be CSI.

In addition, we tested the SSR phenotypes of *surA* and *fkpA* knock-out mutants. Disruption of the *surA* gene results in a considerable decrease in LT-CSS. The *surA* mutation also had an effect on the resistance of CS cells to thermal stress (55°C), acid stress (pH 3.1), and antimicrobial peptide stress (PmB). In fact, the *surA* mutant is even more sensitive to PmB than an *rpoE* mutant, which further supports the argument that *surA* is not controlled by σ^E under our experimental conditions. In contrast, the *surA* mutation had no effect on the H₂O₂ resistance of CS cells. In comparison, the *fkpA* mutation had no effect on LT-CSS, unless it was combined with the *surA* mutation, but *fkpA* did influence cross-resistance to the other stresses tested (Fig. 3; Table 4). Except for the effect of *surA* on LT-CSS, these SSR phenotypes are not as severe as those displayed by the *rpoE* null mutant (Kenyon et al. 2002). Based on these findings, there are likely to be other genes involved in the σ^E -regulated branch of the SSR, and C-starvation appears to cause some type of, as yet uncharacterized, extracytoplasmic stress that acts as a signal for specific activation of the σ^E regulon.

E. coli and *S. Typhimurium* share many metabolic and genetic characteristics. However, previous studies have demonstrated divergence between the σ^E regulon of *E. coli* and that of *S. Typhimurium* (Dartigalongue et al. 2001; Raivio and Silhavy 2001; Rezuchova et al. 2003; Skovierova et al. 2006). In addition, the *rpoE* gene is not essential for the viability of *S. Typhimurium* at any temperature (Humphreys et al. 1999), but in *E. coli* *rpoE* mutants are unable to grow, even at lower temperatures, unless suppressor mutations arise (Hiratsu et al. 1995; De Las Penas et al. 1997; Button et al. 2007). These facts point to major differences in the way *S. Typhimurium* and *E. coli* respond to, and control, cell envelope stress (Rowley et al. 2006).

In comparison, the extracytoplasmic function (ECF) sigma factors of many other bacteria control unique physiological functions. Although many of these ECF σ factors share significant homology to the *E. coli* σ^E , the type of extracytoplasmic stress that they respond to, and the set of genes that they regulate, is often very different (Missiakas and Raina 1998; Raivio and Silhavy 2001; Rowley et al. 2006). In fact, the first ECF sigma factor discovered was AlgU (formerly AlgT) of *Pseudomonas aeruginosa* (Schurr et al. 1996; Xie et al. 1996). In response to extracytoplasmic stress, AlgU directs the transcription of genes involved in the biosynthesis of alginate, an exopolysaccharide that is responsible for the mucoidy phenotype and virulence of this organism (Hershberger et al. 1995; Yu et al. 1995). Examples of other ECF regulons include the CarQ regulon of *Myxococcus xanthus*, involved in regulating carotenoid biosynthesis (Gorham et al. 1996), and the σ^E regulon of *Photobacterium* strain SS9, responsible for the high-pressure resistance of this marine bacterium (Chi and Bartlett 1995).

In conclusion, *surA* and *fkpA* are not σ^E -dependent CSI genes, and thus by definition, are not members of the σ^E -regulated branch of *Salmonella*'s SSR. However, *surA* and *fkpA* are expressed in LP and CS cells in a σ^E -independent manner, and the presence of these periplasmic PPIases contributes to the survival, and possibly recovery, of *Salmonella* cells exposed to nutrient-poor conditions and other types of environmental stress.

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Fig. 1. RNA slot-blot analysis. *surA* mRNA levels in C5 parental and *rpoE* null genetic backgrounds during log-phase (LP) growth and following 5-h and 24-h of C-starvation (CS) in MS minimal media. Blots were developed using the Northern hybridization protocol described by Kenyon et al. (2007)

Fig. 2.

Western blot analysis. SurA protein levels in C5 parental, *rpoE* null and $\Delta surA$ null genetic backgrounds during log-phase (LP), stationary-phase (SP) and C-starvation (5-h and 24-h of CS) in MS minimal media. Westerns were performed as described by Kenyon et al. (2002)

Fig. 3. Comparison of the long-term C-starvation survival (LT-CSS) of *S. Typhimurium* C5 parental, *surA* mutant, *fkpA* mutant, and *surA fkpA* double mutant strains. Cells were starved in MS media with no added glucose (C-source) for a total of 21 days and viability counts determined as described in Methods. Results are reported as the mean values $\pm$ SEM determined from at least three separate experiments. Percent survival is relative to the maximum c.f.u. ml⁻¹ reached for each culture, typically $\sim 5 \times 10^8$ c.f.u. ml⁻¹.

Table 1

Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
Strains		
ST251	Mouse-virulent <i>S. Typhimurium</i> strain C5	Humphreys et al. (2003)
ST252(BRD1115)	C5 Δ <i>surA</i>	Sydenham et al. (2000)
ST253 (GVB844)	C5 <i>fkpA</i> :: Ω -Km ^r	Humphreys et al. (2003)
ST254 (GVB845)	C5 Δ <i>surA fkpA</i> :: Ω -Km ^r	Humphreys et al. (2003)
SMS754	C5 Δ <i>surA</i> /pKS7	This study
ST255	C5 <i>fkpA</i> :: Ω -Km ^r /pSH133	This study
SMS798	C5/pRS1274	This study
SMS792	C5/pKS15	This study
SMS790	C5/pKS18	This study
SMS807	C5/pKS22	This study
SMS782	C5 <i>rpoE</i> ::Cm ^r	This study
SMS853	C5 <i>rpoE</i> ::Cm ^r /pRS1274	This study
SMS854	C5 <i>rpoE</i> ::Cm ^r /pKS15	This study
SMS855	C5 <i>rpoE</i> ::Cm ^r /pKS18	This study
SMS856	C5 <i>rpoE</i> ::Cm ^r /pKS22	This study
ST41	Mouse-virulent <i>S. Typhimurium</i> strain SL1344 (<i>hisG46</i>)	Hoiseth and Stocker (1981)
ST213 (GVB311)	SL1344 <i>rpoE</i> :: Ω -Km ^r	Humphreys et al. (1999)
SMS758	SL1344/pRS1274	This study
SMS894	SL1344/pKS30	This study
SMS895	ST213/pKS30	This study
Plasmids		
pWSK29	Low-copy-number vector (Ap ^r)	Wang and Kushner (1991)
pKS7	pWSK29- <i>surA</i> (Ap ^r)	This study
pSH133	pWSK29- <i>fkpA</i> (Ap ^r)	This study
pRS1274	Vector for transcriptional <i>lacZ</i> fusions (Ap ^r)	Simons et al. (1987)
pKS15	pRS1274- <i>impP</i> :: <i>lacZ</i> (Ap ^r)	This study
pKS18	pRS1274- <i>surAP</i> :: <i>lacZ</i> (Ap ^r)	This study
pKS22	pRS1274- <i>fkpAP</i> :: <i>lacZ</i> (Ap ^r)	This study
pKS30	pRS1274- <i>rpoEP</i> :: <i>lacZ</i> (Ap ^r)	This study

Ap^r ampicillin resistance, Cm^r chloramphenicol resistance, Km^r kanamycin resistance

Table 2

Oligonucleotide primers used in this study

Primer purpose and name	Sequence (5'-3')
Construction of complementation plasmids using pWSK29	
PR26 (<i>surA</i> -R)	gccgggagtgataacaacg
PR27 (<i>surA</i> -F)	acattctgccgtaccaaacg
fkpA3 (<i>fkpA</i> -F)	ctgatatcctgcatgatgtacc
fkpA4 (<i>fkpA</i> -R)	gcaaatcaaattatggtgagtaatat
Construction of promoter- <i>lac</i> fusions in pRS1274	
PR33 (<i>impP</i> -R)	gccagaagagtgggaatac
PR34 (<i>impP</i> -F)	ggcgacgccaattatctt
PR27 (<i>surA</i> -F)	acattctgccgtaccaaacg
PR84 (<i>surAP</i> -R)	gcagcggtttccagttcttc
PR87 (<i>fkpAP</i> -R)	cagtgtttcatccatctcca
PR88 (<i>fkpAP</i> -F)	tgcatgatgtacctcttatgttg
PR166 (<i>rpoEP</i> -F)	gcgagagataaacggcaaaa
PR167 (<i>rpoEP</i> -R)	ccccaaccaaatttcac
Preparation of DIG-labeled probe for RNA slot-blot	
PR100 (' <i>surA</i> '-F2)	cattgttgaagaagcgcgta
PR106 (' <i>surA</i> '-R3)	cggtcacggagatactctgg

Table 3
Activity of promoter-*lac* transcriptional fusions in LP and CS cells of *Salmonella enterica* serovar Typhimurium

Transcriptional fusions	Parental background			<i>rpoE</i> null background		
	LP	5-h CS	24-h CS	LP	5 h-CS	24-h CS
<i>impP::lac</i>	10,771.5 ± 849.4	9,978.9 ± 2,088.1	7,890.9 ± 1,302.5	14,036 ± 727.6	12,560.4 ± 460.6	7,416.0 ± 426.5
<i>surAP::lac</i>	296.0 ± 56.6	310.7 ± 59.4	246.6 ± 48.3	596.4 ± 67.2	630.5 ± 123.3	538.6 ± 143.6
<i>fkpAP::lac</i>	3,551.6 ± 480.9	3,653.7 ± 504.5	2,932.6 ± 292.7	7,195.9 ± 648.0	6,712.5 ± 936.3	5,282.3 ± 1,063.3
<i>rpoEP::lac</i>	6,270.9 ± 1,255.1	8,335.3 ± 2,039.1	9,055.5 ± 1,207.9	6,627.6 ± 330.2	6,702.1 ± 86.2	5,430.3 ± 136.7

Plasmids pKS15, pKS18 and pKS22, carrying the *imp*, *surA* and *fkpA* promoter fusions, respectively, were in either the C5 parental or *rpoE*::Cm^r background. Plasmid pKS30, carrying the *rpoE* promoter fusion, was in either the SL1344 parental or *rpoE*::Km^r backgrounds. LP and CS cells were grown as described in Materials and Methods. Results are reported as Miller units of β-galactosidase activity (Miller 1992) determined from at least three separate experiments ± SEM

Table 4

Effects of *surA* and *fkpA* knock-out mutations on the cross-resistance of LP and CS cells of *Salmonella enterica* serovar Typhimurium

Type of challenge and strain (number and pertinent genotype)	LP cells	5-h CS cells	24-h CS cells
55°C for 16 min			
ST251 (C5 parent)	UD	20.1 ± 5.2	51.3 ± 5.2
ST252 (Δ <i>surA</i>)	UD	1.7 ± 1.0	6.9 ± 4.0
SMS754 (Δ <i>surA</i> /pKS7)	UD	9.6 ± 11.3	23.0 ± 11.2
ST253 (<i>fkpA</i> ::Km ^r)	UD	0.42 ± 0.31	1.6 ± 0.62
ST255 (<i>fkpA</i> ::Km ^r /pSH133)	UD	11.5 ± 2.5	30.7 ± 10.8
ST254 (Δ <i>surA</i> <i>fkpA</i> ::Km ^r)	UD	0.13 ± 0.15	1.4 ± 1.6
pH 3.1 for 40 min			
ST251 (C5 parent)	3.6 ± 2.3	44.5 ± 8.2	18.2 ± 11.2
ST252 (Δ <i>surA</i>)	0.33 ± 0.15	25.8 ± 9.2	0.71 ± 0.25
SMS754 (Δ <i>surA</i> /pKS7)	0.56 ± 0.24	54.6 ± 17.8	33.1 ± 20.6
ST253 (<i>fkpA</i> ::Km ^r)	0.18 ± 0.081	20.0 ± 11.7	0.23 ± 0.18
ST255 (<i>fkpA</i> ::Km ^r /pSH133)	0.071 ± 0.067	51.3 ± 9.0	4.0 ± 2.5
ST254 (Δ <i>surA</i> <i>fkpA</i> ::Km ^r)	0.28 ± 0.17	4.1 ± 1.2	0.0063 ± 0.0065
15 mM H ₂ O ₂ for 40 min			
ST251 (C5 parent)	UD	20.9 ± 15.8	46.7 ± 17.8
ST252 (Δ <i>surA</i>)	UD	10.5 ± 0.91	39.3 ± 2.2
ST253 (<i>fkpA</i> ::Km ^r)	UD	16.1 ± 7.9	45.3 ± 13.7
ST254 (Δ <i>surA</i> <i>fkpA</i> ::Km ^r)	UD	19.9 ± 6.9	32.3 ± 11.4

Values shown are the mean percent survival ± SEM determined from at least three separate experiments. Percent survival is relative to the initial c.f.u. ml⁻¹, typically ~5 × 10⁶ c.f.u. ml⁻¹. UD indicates percent survival was undetectable because c.f.u. ml⁻¹ was below the limit of detection