Small outer-membrane lipoprotein, SmpA, is regulated by σ^E and has a role in cell envelope integrity and virulence of *Salmonella enterica* serovar Typhimurium

Claire Lewis,¹ Henrieta Skovierova,² Gary Rowley,³
Bronislava Rezuchova,² Dagmar Homerova,² Andrew Stevenson,¹
Aileen Sherry,¹ Jan Kormanec² and Mark Roberts¹

Correspondence

Mark Roberts
m.roberts@vet.gla.ac.uk

¹Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK

²Institute of Molecular Biology, Slovak Academy of Science, Dubravska cesta 21, 845 51 Bratislava, Slovak Republic

³School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

SmpA is a small outer-membrane lipoprotein that is a component of the essential YaeT outer-membrane protein assembly complex. In *Salmonella enterica* serovar Typhimurium (S. Typhimurium), expression of the *smpA* gene was shown to be directed by two promoters, *smpAp1* and *smpAp2*. The more distal promoter, *smpAp1*, is dependent upon the extracytoplasmic stress response sigma factor σ^E . An *smpA* null mutant was constructed in S. Typhimurium SL1344 and was shown to be more sensitive than its wild-type parent to growth at high temperature and in the presence of sodium cholate, SDS plus EDTA, and the hydrophobic antibiotic rifampicin. The lack of SmpA in S. Typhimurium elicits a σ^E -dependent stress response. These findings are indicative of altered outer-membrane integrity in the *smpA* mutant, probably due to a defect in outer-membrane protein biogenesis. SmpA was not important for entry or survival within murine macrophages; however, the S. Typhimurium *smpA* mutant was attenuated in mice by both the oral and parenteral routes of infection, and SmpA appeared to be most important for the growth of S. Typhimurium at systemic sites.

Received 24 July 2007
Revised 29 October 2007
Accepted 23 November 2007

INTRODUCTION

Salmonellae are intracellular Gram-negative pathogenic bacteria causing a wide spectrum of diseases in vertebrates. *Salmonella enterica* serovar Typhimurium (S. Typhimurium) causes gastroenteritis in humans and other hosts and a systemic typhoid-like disease in mice. Enteric pathogens such as *Salmonella* are exposed to a variety of stressful environments during their life cycle in mammalian hosts. Their cell envelope directly interfaces with the host in the course of infection and is a major target of various host defence systems. To overcome these adverse conditions, bacteria have evolved a number of stress-response systems,

including so-called envelope stress responses (ESRs), which are governed by at least three partially overlapping signal transduction systems, the CpxRA and BaeSR two-component systems, and the extracytoplasmic function (ECF) sigma factor σ^E (Rowley *et al.*, 2006).

In *Salmonella* and *E. coli*, the *rpoE* gene encoding σ^E is located in an operon *rpoE*, *rseA*, *rseB*, *rseC*. The activity of σ^E and its gene is tightly regulated. Under non-stress conditions, σ^E is inactive because it is sequestered by its specific membrane-bound anti-sigma factor RseA. In response to perturbations in outer-membrane protein (OMP) folding elicited by various stress conditions, RseA is cleaved by the successive action of two membrane proteases, DegS and YaeL (RseP), liberating the complex into the cytoplasm, where RseA is degraded to release σ^E (Alba & Gross, 2004). Liberated σ^E can associate with core RNA polymerase to govern the expression of various genes of the σ^E regulon; these include *rpoE* itself, genes that encode proteins involved in cell envelope biogenesis and homeostasis, as well as many genes of unknown function

Abbreviations: ECF, extracytoplasmic function; ESR, envelope stress response; i.p., intraperitoneal; OMP, outer-membrane protein; TSP, transcriptional start point; WT, wild-type.

Supplementary figures showing a comparison of the *smpA* promoter regions in the genera of the *Enterobacteriales* and an amino acid sequence alignment of SmpA/OmlA homologues of representatives of the three phylogenetic classes of proteobacteria are available with the online version of this paper.

(Dartigalongue *et al.*, 2001; Kabir *et al.*, 2005; Rezuchova *et al.*, 2003; Rhodius *et al.*, 2005; Skovierova *et al.*, 2006). In *E. coli*, *rpoE* is an essential gene, but this is not the case for many other bacteria, including *Salmonella*.

S. Typhimurium σ^E is required for oxidative stress resistance, stationary phase survival and pathogenicity. The virulence of *S. Typhimurium* *rpoE* mutants is highly attenuated in mouse models of infection and σ^E is critical for survival of *S. Typhimurium* in macrophages (Humphreys *et al.*, 1999; Testerman *et al.*, 2002). Moreover, σ^E is also essential for resistance to non-oxidative mammalian host defence mechanisms such as antimicrobial peptides (Crouch *et al.*, 2005; Humphreys *et al.*, 1999; Kenyon *et al.*, 2002; Testerman *et al.*, 2002). Therefore, some of the genes of the σ^E regulon should also play a role in pathogenicity.

We have recently identified 62 genes dependent upon σ^E in *S. Typhimurium* (Skovierova *et al.*, 2006). In addition to many genes common to the *E. coli* σ^E regulon with a known or suspected role in envelope homeostasis, several genes specific to *S. Typhimurium* were identified. Also, in both organisms, there are still many genes of unknown function (Dartigalongue *et al.*, 2001; Kabir *et al.*, 2005; Rezuchova *et al.*, 2003; Rhodius *et al.*, 2005; Skovierova *et al.*, 2006).

A number of salmonella envelope proteins are involved in pathogenesis; these envelope proteins or their genes could be targeted in the design of new vaccines and therapeutics. Therefore, we have been investigating the functions of these identified members of the σ^E regulon with an emphasis on their role in pathogenesis. In the present report, we focus

on small membrane protein A (SmpA) and its role in the cell envelope integrity and virulence of *S. Typhimurium*. We have previously found that the *smgA* gene is a member of the σ^E regulon in *E. coli* (Rezuchova *et al.*, 2003), and this was recently independently confirmed (Rhodius *et al.*, 2005). However, we did not identify *smgA* using a similar screening procedure for *S. Typhimurium* σ^E -regulated genes (Skovierova *et al.*, 2006).

Until recently, almost nothing was known about the function of SmpA in *E. coli* and related bacteria. Homologous genes are present in several currently sequenced genomes of Gram-negative bacteria (see below). The SmpA homologue OmlA is an outer-membrane lipoprotein in the opportunistic Gram-negative pathogen *Pseudomonas aeruginosa*, where it is proposed to have a structural role in maintaining cell envelope integrity (Ochsner *et al.*, 1999). Very recently, a comprehensive genetic and biochemical analysis revealed a role for SmpA as a novel structurally and functionally important, but non-essential, member of the multi-component YaeT OMP assembly complex (Sklar *et al.*, 2007). In the present study we characterize the regulation of *smgA* in *S. Typhimurium*, construct an *S. Typhimurium* *smgA* null mutant, and investigate the role of SmpA in cell envelope integrity and *S. Typhimurium* virulence.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were grown in Luria-Bertani (LB), minimal M9 medium or on LB agar (LA) plates (Miller, 1972). M9 medium was supplemented with

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics*	Reference or source
Strains		
<i>S. Typhimurium</i> SL1344	<i>his</i> , mouse-virulent	Hoiseth & Stocker (1981)
<i>S. Typhimurium</i> GVB311	SL1344 <i>rpoE</i> ::Km ^R	Humphreys <i>et al.</i> (1999)
<i>S. Typhimurium</i> GVB1741	SL1344 <i>smgA</i> ::Km ^R	This study
<i>S. Typhimurium</i> GVB2413	SL1344 <i>smgA</i> ::Km ^R pAC-smpA2	This study
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hrdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q ZAM15 Tn10 (Tc ^R)]	Stratagene
Plasmids		
pACYC184	Cm ^R , Tc ^R , low-copy cloning vector	Biolabs
pAC7	Cm ^R , low-copy expression vector with P _{BAD} promoter	Rezuchova & Kormanec (2001)
pAC-rpoEST4	Cm ^R , pAC7 containing <i>S. Typhimurium</i> <i>rpoE</i> gene under the P _{BAD} control	Miticka <i>et al.</i> (2003)
pAC-smpA2	Cm ^R , pACYC184 containing <i>S. Typhimurium</i> <i>smgA</i> gene including <i>smgA1</i> , -2	This study
pKD4	Ap ^R , pANT-S γ derivative containing an FRT-flanked Km ^R	Datsenko & Wanner (2000)
pKD46	Ap ^R , pINT-ts derivative containing <i>araC</i> -P _{araB} and γ , β , <i>exo</i> genes	Datsenko & Wanner (2000)
pTL61T	Ap ^R , low-copy promoterless <i>lacZ</i> fusion vector	Linn & St Pierre (1990)
prpoEP3	Ap ^R , pTL61T containing σ^E -dependent <i>S. Typhimurium</i> <i>rpoEp3</i> promoter	Miticka <i>et al.</i> (2003)

*Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance.

0.2 % glucose, 1 mM MgSO₄ and 0.2 mM histidine. When required, the media were supplemented with 100 µg ampicillin ml⁻¹, 40 µg chloramphenicol ml⁻¹, 50 µg gentamicin ml⁻¹ or 50 µg kanamycin ml⁻¹.

Sensitivity of bacterial strains to toxic agents. Disc diffusion assays. The sensitivity of *Salmonella* strains to toxic agents was measured by disc diffusion assay, as described previously (Humphreys *et al.*, 1999). The MIC of compounds towards the strains was determined as described by Sklar *et al.* (2007).

Isolation of RNA and S1-nuclease mapping. RNA was isolated and S1-mapping was carried out essentially as previously described (Kormanec, 2001; Miticka *et al.*, 2003; Skovierova *et al.*, 2006). Briefly, an overnight culture was diluted 500-fold into fresh LB and incubated at 37 °C with aeration to the exponential (OD₆₀₀=0.5) and stationary phases (OD₆₀₀=1.7). Heat-shock- and cold-shock-stressed cells were grown to exponential phase and subjected to 30 min at 45 °C or 60 min at 10 °C, respectively. For artificial *rpoE* expression, *S. Typhimurium* SL1344 containing pAC-rpoEST4 or pAC7 was grown in LB with chloramphenicol to early exponential phase (OD₆₀₀=0.24) and expression of *rpoE* was induced for 3 h with 0.2 % arabinose.

At the appropriate time point, the *S. Typhimurium* culture was chilled, washed with diethylpyrocarbonate (DEPC)-treated ice-cold 0.15 M NaCl, and total RNA was prepared essentially as described previously (Kormanec, 2001). High-resolution S1-nuclease mapping was performed according to Kormanec (2001). RNA samples (40 µg) were hybridized to 0.02 pmol of appropriate DNA probe labelled at the 5' end with [γ -³²P]ATP and treated with 120 U S1-nuclease (Promega). The probe (a 487 bp DNA fragment) was prepared by PCR amplification from the chromosomal DNA of *S. Typhimurium* SL1344 using the 5' end-labelled reverse primer smpARev (5'-GCGGTCAACATCAGGAGTACTGC-3') and the direct primer smpADir (5'-CGAGGTCGATGTTGGCATCAGC-3'). The SmpARev primer was labelled at the 5' end with [γ -³²P]ATP [CN; 4500 Ci mmol⁻¹ (167 TBq)] and T4 polynucleotide kinase (Promega), as described in Ausubel *et al.* (1995). The protected DNA fragments were analysed on DNA sequencing gels together with G + A and T + C chemical sequencing ladders derived from the end-labelled fragment (Maxam & Gilbert, 1980).

β -Galactosidase assays. Overnight cultures of the *S. Typhimurium* strains with plasmid prpoEP3 containing the σ^E -dependent *rpoEp3* promoter upstream of the *lacZ* reporter gene in pTL61T (Miticka *et al.*, 2003) were diluted 500-fold into 50 ml fresh LB medium with ampicillin, grown at 37 °C with aeration and assayed at hourly intervals. β -Galactosidase assays were performed in triplicate (Miller, 1972).

Construction of an *smpA* mutant. An *smpA* mutant was constructed in *S. Typhimurium* SL1344 using a λ Red mutagenesis system (Datsenko & Wanner, 2000; Rowley *et al.*, 2005). To decrease background, the template plasmid was digested with *Hind*III and an agarose gel-purified 1635 bp DNA fragment was used for PCR with mutagenesis primers smpAFw (5'-ATCACTATGCGCTGTAAAAC-GCTGACTGCTGCCGACGAGGTGTAGGCTGGAGCTGCCTTC-3') and smpARv (5'-ACAAAATTACTTCGTCAACGCCGGTTGTGTTCAATATTGCATATGAATATCCTCCTTAG-3') to amplify the kanamycin-resistance gene cassette with homologous flanking regions from the start and end of the *smpA* gene. The amplified DNA fragment was column-purified (Qiagen) and electroporated into *S. Typhimurium* SL1344 pKD46. Deletion of *smpA* was confirmed by PCR using primers which are external to the site of mutagenesis, smpADir (5'-CGAGGTCGATGTTGGCATCAGC-3') and smpARev2 (5'-GTGTGAAAGCCGTACCACACC-3'), as well as primers k1

(5'-CAGTCATAGCCGAATAGCCT-3') and k2 (5'-CGGTGCCCTG-AATGAACTGC-3') that amplify the kanamycin cassette in order to confirm the orientation of the marker (Datsenko & Wanner, 2000). P22 HT transduction was used to transfer the *smpA* mutation into a clean SL1344 background.

The *S. Typhimurium* SL1344 *smpA* mutant strain was named GVB1741. To complement the *smpA* mutation, *S. Typhimurium* GVB1741 was transformed with plasmid pAC-smpA2 containing the *S. Typhimurium* *smpA* gene including both promoters. The pAC-smpA2 plasmid was constructed by cloning a 780 bp DNA fragment, prepared by PCR amplification using the primers smpADir (5'-CGAGGTCGATGTTGGCATCAGC-3') and SmpAHind (5'-GGGG-GAAGCTTCACAAAATTACTTCGTCAAC-3') and *S. Typhimurium* SL1344 chromosomal DNA as a template, blunt-ended by the Klenow fragment of DNA polymerase and cut with *Hind*III, in a low-copy-number plasmid pACYC184 digested with *Hind*III and *Eco*RV.

Infection of murine macrophages. The ability of *S. Typhimurium* strains to invade, and survive and replicate within, the murine macrophage cell line Raw 264.7 was determined using a gentamicin protection assay performed essentially as previously described (Elsinghorst, 1994; Humphreys *et al.*, 1999; Sydenham *et al.*, 2000). Briefly, an m.o.i. of ~1 : 1 was used, and the number of bacteria inside infected cells was determined at 3 and 24 h post-infection using a gentamicin protection assay.

Analysis of virulence. For all *in vivo* studies, strains were grown statically overnight at 37 °C, centrifuged, washed and resuspended to the appropriate concentration in sterile PBS (pH 7.2), and administered to mice in doses of 200 µl. Female BALB/c mice (6–8 weeks old; Harlan) were used throughout. Virulence was assessed initially by intraperitoneal (i.p.) infection using a competition index assay, as previously described (Beuzon & Holden, 2001; Humphreys *et al.*, 2003; Rowley *et al.*, 2005) using three mice per group. For oral infection, five mice per group were used, the inoculum was administered via oral gavage and mice were culled 7 days later. Organs (livers, spleens, Peyer's patches and mesenteric lymph nodes) were isolated and homogenized, and the number of bacteria present was determined by viable counting.

Statistical analysis. The statistical significance of datasets was analysed by two-tailed Student's *t* test or ANOVA, as appropriate.

RESULTS

Transcriptional analysis of *smpA* and its dependence upon σ^E in *S. Typhimurium*

Using an *E. coli* two-plasmid system for the identification of promoters recognized by *S. Typhimurium* σ^E , we have recently identified 34 σ^E -dependent promoters directing the expression of 62 genes that are members of the σ^E regulon in *S. Typhimurium* (Skovierova *et al.*, 2006). However, several σ^E -dependent promoters previously identified in *E. coli* using the same system were not found by this screen in *S. Typhimurium*, among them *smpAp* (Rezuchova *et al.*, 2003).

Therefore, in order to investigate the expression of the *S. Typhimurium* *smpA* gene and to determine whether it is regulated by σ^E , high-resolution S1-nuclease mapping was performed using the 5'-labelled S1 probe (Fig. 1a). RNA was isolated from *S. Typhimurium* SL1344 and its isogenic

Fig. 1. Transcriptional analysis of the *smpA* gene in *S. Typhimurium*. (a) Genetic organization of the *S. Typhimurium smpA* region. The genes are indicated by thick arrows. The thin line below the map represents a DNA fragment (5' end-labelled at the end marked with an asterisk) that was used as a probe in the S1-nuclease mapping experiment. The positions of the identified promoters are indicated by bent arrows. (b) High-resolution S1-nuclease mapping of the TSPs for the *S. Typhimurium* promoters directing expression of the *smpA* gene. The 5'-labelled DNA probe hybridized in parallel with 40 µg RNA and was treated with 120 U S1-nuclease (Methods). RNA was isolated from the indicated *S. Typhimurium* strain grown in LB medium. Lanes: 1, exponential phase; 2, heat shock; 3, cold shock; 4, stationary phase; C, *E. coli* tRNA as a control. The other RNAs were isolated from *S. typhimurium* SL1344 containing pAC-rpoEST4 (lane 5) or pAC7 (lane 6) grown to early exponential phase and induced for 3 h with 0.2% arabinose. The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G + A (lane A) and T + C (lane T) sequencing ladders derived from the end-labelled fragments (Kormanec, 2001). Thin horizontal arrows indicate the positions of RNA-protected fragments and thick bent vertical arrows indicate the nucleotides corresponding to the TSPs of the indicated promoters. Before assigning the TSP, 1.5 nt was subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from primer extension products and the chemical sequencing reactions. All S1-nuclease mapping experiments were performed twice using independent sets of RNA with similar results. (c) Nucleotide sequence of the *S. Typhimurium smpA* promoter region. The deduced protein products corresponding to the *recN* and *smpA* genes are given in single-letter amino acid code in the second position of each codon. The initiation and termination codons are underlined. The TSPs of the promoters are indicated by bent arrows. The proposed -10 and -35 boxes of the promoters are in bold type and underlined. The numbers correspond to the nucleotide positions, which refer to the GenBank/EMBL/DDBJ accession number AE006468.

rpoE mutant *S. Typhimurium* GVB311 from different growth phases and under stress conditions that have previously been shown to induce the expression of σ^E -dependent promoters (Miticka *et al.*, 2003). RNA was also isolated from *S. Typhimurium* SL1344 containing pAC-rpoEST4 (which has the *S. Typhimurium rpoE* gene under the control of the arabinose-inducible P_{BAD} promoter) induced with arabinose. As shown in Fig. 1(b), an RNA-protected fragment was identified that corresponded to the *smpAp1* promoter with a transcriptional start point (TSP) in a position identical to that of the σ^E -dependent *E. coli smpAp* promoter (Rezuchova *et al.*, 2003; Rhodius *et al.*, 2005). An identical RNA-protected fragment was also identified using rRNA isolated from *S. Typhimurium* SL1344 pAC-rpoEST4 (Fig. 1b, lane 5) but not RNA from *S. Typhimurium* SL1344 pAC7 (the control vector) (Fig. 1, lane 6) or with control rRNA (Fig. 1b, lane C). The *smpAp1* promoter was induced in stationary phase and by cold shock, conditions previously shown to induce σ^E -dependent promoters in *S. Typhimurium* (Miticka *et al.*, 2003). However, when RNA from the same conditions was prepared from the *S. Typhimurium rpoE* mutant, no RNA-protected fragment was identified that corresponded to the *smpAp1* promoter (Fig. 1b). These results clearly indicated that this promoter is dependent upon σ^E in *S. Typhimurium*. The sequence of the *smpAp1* promoter (Fig. 1c) was highly similar to the σ^E consensus sequence GGAAGTT-N₁₅-GTCTAA (Skovierova *et al.*, 2006). Downstream of the σ^E -dependent *smpAp1* promoter (and upstream of the *smpA* gene), we identified a second, σ^E -independent, promoter, *smpAp2*, expression of which in both the wild-type (WT) and *rpoE* strains was almost constitutive during exponential growth, with a partial decrease after heat shock and in stationary phase (Fig. 1b). Based on its sequence (Fig. 1c), it is probably recognized by RNA polymerase containing the principal sigma factor σ^{70} , as it is similar to its consensus sequence, TTGACA-N₁₆₋₁₈-TATAAT (Pribnow, 1975). In *E. coli*, in addition to the σ^E -dependent *smpAp1* promoter that we previously identified (Rezuchova *et al.*, 2003), we also identified a σ^E -independent promoter with a TSP in a position similar to that of *S. Typhimurium smpAp2* (data not shown). Thus, expression of *smpA* in both *S. Typhimurium* and *E. coli* is governed by two promoters, of which one, *smpAp1*, is dependent upon σ^E .

Comparison of the *smpA* promoters between *E. coli* and *S. Typhimurium* revealed that both are conserved in both strains, having an almost identical σ^E consensus sequence for the σ^E -dependent promoter (one mismatch in the -10 region), and identical -10 and -35 regions for the σ^E -independent promoters (Supplementary Fig. S1). Thus, it is likely that regulation of *smpA* is similar in both strains.

Characterization of SmpA, and its gene and promoter, in other bacteria

SmpA belongs to the bacterial outer-membrane lipoprotein family SmpA/OmlA (pfam04355). Using a BLAST search of

all databases and currently sequenced genomes (759 bacterial, 41 archaeal and 135 eukaryotic) we found homologues in Gram-negative proteobacteria only, and then only in the α , β and γ classes. Comparison of *S. Typhimurium* SmpA with several selected homologues revealed the highest similarity to members of the γ -proteobacteria. As for *S. Typhimurium* SmpA, all the homologues contain a lipobox motif with a conserved Cys residue at position 1 that is covalently modified with a lipid moiety. Based on the rules for amino acids in second and third positions (Narita *et al.*, 2004), they should all be outer-membrane lipoproteins (Supplementary Fig. S2). Interestingly, although SmpA homologues are present in the α -, β - and γ -proteobacteria, the chromosomal context of the *Salmonella* and *E. coli* gene order *recN*, *smpA*, *yjfF*, *yjfG* (Fig. 1a) is conserved in γ -proteobacterial orders *Enterobacteriales* and *Vibrionales* only.

The *smpA* promoter region is identical in all six currently sequenced *S. enterica* serovars. There are only four mismatches in the non-essential promoter regions in *Salmonella bongori*. Comparison of the *smpA* promoter regions among members of *Enterobacteriales* (Supplementary Fig. S1) has revealed that they all contain a sequence highly similar to the σ^E -dependent *smpAp1* promoter, indicating that in all the strains *smpA* is likely under the control of σ^E . However, the similarity of the σ^E -independent *smpAp2* promoter is lower and is mainly conserved in the closely related *Enterobacteriales* genera *Salmonella*, *Escherichia* and *Shigella*.

Construction of an *S. Typhimurium smpA* mutant and its phenotypic analysis

Using λ Red mutagenesis (Datsenko & Wanner, 2000), we replaced the coding region of the *smpA* gene in *S. Typhimurium* SL1344 with a kanamycin-resistance cassette to generate strain GVB1741. The mutation did not affect colony morphology and growth in liquid media at temperatures ranging from 25 to 42 °C (data not shown). However, the *smpA* mutant grew less well than the WT strain at high temperature (46 °C) (Fig. 2a). The means of the optical densities of the two strains were significantly different ($P < 0.05$) from 7 h onwards. This effect was complemented by plasmid pAC-smpA2, which encodes a copy of the *S. Typhimurium smpA* gene with both promoters (Fig. 2a).

Mutation of the *smpA* homologue *omlA* increases the susceptibility of *P. aeruginosa* to several detergents and antibiotics (Ochsner *et al.*, 1999). We therefore investigated the sensitivity of the *S. Typhimurium smpA* mutant to a variety of small toxic compounds and detergents by a similar disc diffusion method on solid agar medium. As shown in Table 2, at the concentrations tested, none of the detergents tested inhibited the growth of either the WT or *smpA* strains, which is in contrast to the phenotype of a *P. aeruginosa omlA* mutant (Ochsner *et al.*, 1999). The *smpA* mutant was not more sensitive than the WT strain to

Fig. 2. Effect of inactivation of *smpA* on growth of *S. Typhimurium* under stress and activation of the σ^E system. *S. Typhimurium* SL1344 (WT), GVB1741 (*smpA* mutant) and GVB2413 (*smpA* pAC-*smpA2*) were grown in LB medium at 46 °C (a), or in LB medium with 1 % SDS and 1 mM EDTA at 37 °C (b). Growth was monitored by measuring OD₆₀₀. Each value represents the mean of triplicate experiments. (c) The activity of the σ^E stress response was measured by using the σ^E -dependent *rpoEp3-lacZ* fusion plasmid prpoEP3. β -Galactosidase activities were determined in the indicated strains containing prpoEP3 during growth in LB medium. Bars show the mean from triplicate experiments; error bars show SD.

in agreement with the increased rifampicin sensitivity of an *E. coli smpA* mutant (Sklar *et al.*, 2007). The *E. coli smpA* mutant also exhibits increased sensitivity to cholate, and it is unable to grow on medium containing 0.5 % SDS plus 1 mM EDTA (Sklar *et al.*, 2007). Similarly, the *S. Typhimurium smpA* mutant exhibited increased sensitivity to sodium cholate (MIC 12.5 mg ml⁻¹ for WT and *smpA* pAC-*smpA2*, and 10 mg ml⁻¹ for the *smpA* mutant), although not to such an extent as the *E. coli* mutant. However, in contrast to the results in *E. coli*, the *smpA* mutation had little effect on the growth of *S. Typhimurium* in LB medium with 0.5 % SDS and 1 mM EDTA. When a higher concentration of SDS (1.0 %) and 1 mM EDTA were used, the *smpA* mutant grew slightly slower and lysed more rapidly than the WT strain after prolonged cultivation (Fig. 2b). The differences in the means of the optical densities of the *smpA* cultures versus the WT and *smpA* pAC-*smpA2* cultures were statistically significant ($P < 0.05$) at all time points post 4 h.

After 24 h culture in 1 % SDS + 1 mM EDTA, survival of the *smpA* mutant was 50 % that of the WT strain (determined by viable counting). All the mutant phenotypes were complemented by plasmid pAC-*smpA2* containing a functional copy of the *S. Typhimurium smpA* gene, including both *smpA**p1* and -*p2* promoters (Table 2; Fig. 2a, b).

Loss of SmpA activates the σ^E -mediated envelope stress response

Defects in OMP folding and localization have been found to trigger the σ^E stress response (Alba & Gross, 2004; Rowley *et al.*, 2006). As SmpA has recently been found to be a part of the YaeT complex essential for assembly of OMPs (Sklar *et al.*, 2007), we tested whether the absence of SmpA from this complex elicits a σ^E stress response in *S. Typhimurium*. We have previously shown that expression of the auto-regulated σ^E -dependent promoter *rpoEp3* is dramatically induced at stationary phase in *S. Typhimurium*, and that its activity corresponds to the level of σ^E , thus representing a sensitive marker for the σ^E stress response (Miticka *et al.*, 2003). Therefore, the *rpoEp3-lacZ* transcriptional fusion plasmid prpoEP3 (Miticka *et al.*, 2003) was transformed into

chloramphenicol, gentamicin or polymyxin B, but it was slightly more sensitive to H₂O₂. However, compared to the WT strain, the *smpA* mutant was more sensitive to the hydrophobic antibiotic rifampicin (Table 2). The MIC of rifampicin for the *smpA* mutant (12.5 μ g ml⁻¹) was half that of the WT strain and the *smpA* mutant harbouring the complementing plasmid pAC-*smpA2* (25 μ g ml⁻¹). This is

Table 2. Sensitivity of *S. Typhimurium* WT and *smpA* mutant strains to various detergents and toxic compounds

Compound*	Zone of growth inhibition (mm)†		
	SL1344	GVB1741	GVB1741 + pAC-smpA2‡
20 % SDS	0	0	0
20 % Deoxycholate	0	0	0
10 % Tween-20	0	0	0
10 % Triton X-10	0	0	0
0.5 M EDTA	0	0	0
2 % Nalidixic acid	18	18	18
0.4 % Chloramphenicol	6	6	0§
0.2 % Gentamicin	12	12	12
0.2 % Polymyxin B	8	8	8
0.2 % Rifampicin	6	12	6
3 % H ₂ O ₂	12	13	12
2 % Paraquat	16	16	16

*A solution of the particular compound (7 µl) was spotted on a sterile filter disc and placed on M9 top agar containing 10⁶ c.f.u.

†The zones were measured after 24 h at 37 °C and are the means of three determinations.

‡pAC-smpA2 is a complementing plasmid containing the *S. Typhimurium smpA* gene and both *smpA* promoters (Table 1).

§pAC-smpA2 confers chloramphenicol resistance.

the *S. Typhimurium* WT and *smpA* mutant strains, and the level of β -galactosidase was measured during growth in LB medium. As shown in Fig. 2(c), the activity of the σ^E -dependent *rpoEp3* promoter was more than twofold higher in the *smpA* mutant than the WT strain at early exponential phase, and this increase persisted at all stages of growth. These results indicated that the lack of SmpA elicited a σ^E -dependent envelope stress response in *S. Typhimurium*.

Effect of *smpA* mutation upon *S. Typhimurium* invasion of and survival within a murine macrophage cell line

To investigate whether *smpA* is important for entry and/or survival within macrophages, the *S. Typhimurium smpA* mutant and its isogenic WT and *rpoE* strains were used to infect the macrophage-like cell line RAW264.7, and the number of viable salmonellae present at 3 and 24 h after infection was determined; the results are presented in Fig. 3(a). At both the 3 and 24 h time points the *smpA* mutant was present in macrophages at slightly lower levels than the WT strain; however, these differences were not statistically significant. In contrast, as reported previously, *S. Typhimurium rpoE* mutants have a severe defect in intramacrophage survival (Humphreys *et al.*, 1999; Testerman *et al.*, 2002). This suggests that at least under the conditions tested, *smpA* is not important for invasion of or survival and replication within macrophages.

Fig. 3. Effect of the *smpA* mutation on *S. Typhimurium* infection of macrophages and mice. (a) Infection of macrophages. Bacteria at an m.o.i. of ~1:1 were incubated with RAW 264.7 cells. The assay was performed as described in Methods. The bars show the number of viable bacteria (as a percentage of the initial inoculum) inside the macrophages at 3 h (black bars) and 24 h (grey bars) after infection. Bars show the mean from triplicate experiments; error bars show SD. (b) Oral infection of mice. Mice were challenged orally with either 5×10⁵ c.f.u. of the WT strain or 1.2×10⁷ c.f.u. of the *smpA* mutant, and the number of bacteria present in different organs was determined 7 days later. Bars show the mean of four mice; error bars show SD.

Effect of inactivation of *smpA* on *S. Typhimurium* infection of mice

To determine if the absence of *smpA* affects salmonella virulence, the ability of the *smpA* mutant to compete with the WT strain for growth in the liver and spleens of mice was analysed by a competition assay. A mixed inoculum containing ~1 × 10³ c.f.u. of both the WT and *smpA* strains was given by the i.p. route to mice. A competitive index (CI) of ~1 indicates that the strains are of equal virulence. The assay was performed twice and the CIs ranged from 0.08 to 0.12. In all cases the *smpA* mutant was recovered from the liver and spleens in significantly lower numbers

compared to the input inoculum than the WT strain ($P < 0.05$). We also investigated the role of SmpA during infection via the oral route. Mice were inoculated orally with either 5×10^5 c.f.u. of the WT strain or 1.2×10^7 c.f.u. of the *smpA* mutant, and the number of bacteria present was determined 7 days later (Fig. 3b). We gave a higher dose of the *smpA* mutant because we were concerned that we would not recover c.f.u. of the *smpA* bacteria from the tissues if a dose similar to that of the WT strain was used. The oral LD₅₀ of SL1344 is $\sim 1 \times 10^5$ to 1×10^6 c.f.u. (Humphreys *et al.*, 1999; Monack *et al.*, 2000). Therefore, if the mice were infected with SL1344 at the dose used for the *smpA* mutant, most of the mice would be dead or dying at 7 days.

Similar numbers of c.f.u. of the WT and *smpA* strains were found in the Peyer's patches and mesenteric lymph nodes of infected mice. If the numbers of c.f.u. present in the mesenteric lymph nodes and Peyer's patches were normalized for the c.f.u. in the inoculum, then the result for the *smpA* mutant was significantly lower ($P < 0.05$) than that of the WT strain. Even though the dose of the *smpA* mutant was much higher than that of the WT strain, there were significantly fewer *smpA* than WT bacteria in the liver and spleen ($P < 0.05$).

DISCUSSION

The aim of the present study was to investigate the role of the small outer-membrane lipoprotein SmpA in the cell envelope integrity and virulence of *S. Typhimurium*. We and others have previously shown that the ECF sigma factor σ^E is critically important for the oxidative stress resistance, stationary-phase survival and pathogenicity of *S. Typhimurium* (Crouch *et al.*, 2005; Humphreys *et al.*, 1999; Kenyon *et al.*, 2002; Testerman *et al.*, 2002), indicating that some members of the σ^E regulon may have a role in the virulence of *S. Typhimurium*. We are investigating the σ^E regulon with respect to salmonella virulence. Although *smpA* was not identified in our previous genetic screen for members of the σ^E regulon in *S. Typhimurium* (Skovierova *et al.*, 2006), the present results clearly show that *smpA* belongs to the *S. Typhimurium* σ^E regulon. Expression of *smpA* in *S. Typhimurium* is directed by two promoters. The distal promoter, *smpAp1*, is dependent upon σ^E , and the proximal promoter, *smpAp2*, is probably recognized by σ^{70} . Similar regulation has also been found for its counterpart in *E. coli*, reflecting the high conservation of the *smpA* promoter region in both strains (Supplementary Fig. S1). Interestingly, although SmpA homologues are present in three classes of proteobacteria (α , β and γ), the *smpA* promoter region is conserved only in the γ -proteobacterial order *Enterobacteriales*. The highest similarity was in the region covering the σ^E -dependent promoter. Thus, it is likely that *smpA* is dependent upon σ^E and regulated in a similar way in all these bacteria.

As with *E. coli*, SmpA is not essential for *S. Typhimurium* under standard growth conditions. The growth rate of the

smpA mutant was reduced relative to the WT strain at high temperature (46 °C), although this growth defect was smaller than for an *S. Typhimurium* *rpoE* mutant (Rowley *et al.*, 2005). The *S. Typhimurium* *smpA* mutant showed increased sensitivity to sodium cholate, SDS plus EDTA, and the hydrophobic antibiotic rifampicin. *P. aeruginosa* *omlA* and *E. coli* *smpA* mutants also exhibit increased sensitivity to detergents, although there are differences between the strains regarding the magnitude of this sensitivity. The *P. aeruginosa* *omlA* mutant exhibits a much higher susceptibility to anionic detergents. The *omlA* mutant almost stops growing in 0.1 % SDS (Ochsner *et al.*, 1999). Likewise, an *E. coli* *smpA* mutant does not grow in the presence of 0.5 % SDS plus 1 mM EDTA (Sklar *et al.*, 2007). In contrast, the growth of the *S. Typhimurium* *smpA* mutant was not so strongly affected by detergents. In fact, both WT and *smpA* *S. Typhimurium* strains grew in the presence of 5 % SDS (data not shown). This may reflect the high resistance of *Salmonella* strains to detergents. Despite differences in the magnitude of the effects of loss of SmpA/OmlA, the *in vitro* phenotypes of *E. coli*, *P. aeruginosa* and *S. Typhimurium* mutants are similar, indicating a role for SmpA/OmlA homologues in maintaining the cell envelope integrity in Gram-negative bacteria. This is presumed to be due to their function as a component of the YaeT OMP assembly complex. SmpA has been shown to associate with YfiO and NlpB in the complex, and this interaction is independent of another component, YfgL (Sklar *et al.*, 2007). In *E. coli*, mutations in *nlpB* and *yfgL* also increase sensitivity to hydrophobic antibiotics, bile salts and detergents (Sklar *et al.*, 2007). The two other genes that encode members of the complex, *yaeT* and *yfiO*, are essential in *E. coli* (Wu *et al.*, 2005).

The function or activity of the YaeT complex seems to vary in Gram-negative bacteria. While homologues of the essential YaeT are present in all Gram-negative bacteria (Gentle *et al.*, 2005), the other components of the complex are not so conserved. Examination of available bacterial genomes revealed that YfiO, which is essential in *E. coli*, has no homologues in *Chlamydiae*, *Chlorobi*, *Fusobacteria*, *Planctomycetes*, *Thermotogae*, *Deinococcus-Thermus* and *Spirochaetes*; homologues of the non-essential YfgL are absent in *Chlamydiae*, *Spirochaetes* and ϵ -*Proteobacteria*; and, as for SmpA, homologues of the non-essential NlpB are present only in α -, β - and γ -*Proteobacteria*.

The σ^E -dependent ESR is induced by OMP folding perturbations that are elicited by various stress conditions. The members of the σ^E regulon produced following these stress conditions are involved in the correction of these cell envelope defects (Alba & Gross, 2004; Rowley *et al.*, 2006). As the YaeT complex is essential for assembly of OMPs, flaws in this complex should result in OMP assembly defects which should elicit a σ^E -dependent ESR. We found that this was the case in *S. Typhimurium* using the σ^E -dependent *rpoEp3* promoter fusion as a reporter. In an *E. coli* *smpA* mutant, production of the σ^E -regulated protein HtrA (DegP) is increased 1.5–2-fold relative to the WT

strain, indicating that a σ^E response is activated in this strain (Sklar *et al.*, 2007). Interestingly, all the components of the YaeT complex belong to the σ^E regulon, both in *E. coli* and *S. Typhimurium* (Rhodius *et al.*, 2005; Skovierova *et al.*, 2006; M. Roberts, unpublished results). Likewise, lack of another component of the YaeT complex, YfgL, increases σ^E activity in *E. coli* and *S. enterica* serovar Enteritidis (Fardini *et al.*, 2007; Onufryk *et al.*, 2005).

In contrast to the *S. Typhimurium* *rpoE* mutant, the *smpA* mutation did not significantly affect the ability of *S. Typhimurium* to enter or replicate within macrophages *in vitro* (Fig. 3a). However, the mutant was significantly attenuated in mice by both the oral and parenteral routes of infection. Compared to the WT strain, approximately 10-fold fewer *smpA* bacteria could be isolated from the liver and spleen after parenteral infection of mice, indicating that the *smpA* mutant survived significantly less than the WT strain. Comparison of the ability of the *smpA* mutant and the WT strain to infect mice via the oral route revealed that the *smpA* mutation affected growth/survival of *S. Typhimurium* at systemic sites (spleen and liver) more than at gut-associated sites (Peyer's patches and mesenteric lymph nodes). This could reflect defective replication/survival in systemic tissues or defective translocation of the *smpA* mutant from the mesenteric lymph nodes. Intriguingly, a similar effect has also been found for *S. Typhimurium* strains with mutation in *rpoE*, in the *rpoE*-regulated genes *htrA* and *surA*, and in *degS*, which encodes a protease that regulates σ^E (Humphreys *et al.*, 1999; Rowley *et al.*, 2005; Sydenham *et al.*, 2000). This possibly indicates that members of the σ^E regulon are more important for systemic than enteric *S. Typhimurium* infection.

Although the *S. Typhimurium* *smpA* mutant exhibited relatively mild phenotypes *in vitro* (as does an *E. coli* *smpA* mutant), it exhibited a more severe phenotype *in vivo* and was essential for full salmonella virulence. Therefore, it is possible that the stresses to which the *S. Typhimurium* *smpA* mutant was exposed *in vitro* did not replicate and/or were not as severe as those encountered *in vivo* during murine infection. This shows that the relevance of stress-response genes may not be revealed by *in vitro* studies alone. Interestingly, *smpA* was one of the genes (along with *rpoE*) found to be activated in *S. Typhimurium* during infection of macrophages *in vitro* (Eriksson *et al.*, 2003). Also, *smpA* was identified as one of the most highly expressed genes in the spleens of *S. Typhimurium*-infected mice (Rollenhagen *et al.*, 2004).

Recently, another member of the σ^E regulon and the YaeT complex, YfgL, has been characterized in *S. Enteritidis*. As for the *S. Typhimurium* *smpA* mutant, the *S. Enteritidis* *yfgL* mutant is more sensitive to rifampicin and is greatly attenuated in mice (Fardini *et al.*, 2007). By an unknown mechanism, the *yfgL* mutation negatively affects the expression of genes that encode proteins of the two *S. Enteritidis* type III secretion systems (TTSS-1 and TTSS-2)

(Charlson *et al.*, 2006). We found no difference in the production and secretion of the TTSS-1 protein SipC and the TTSS-2 protein SseC by the WT and *smpA* mutant strains (data not shown).

These σ^E -dependent YaeT complex proteins may be good targets for anti-salmonella therapy, and *Salmonella* strains with mutations in these genes may be live vaccine candidates. Experiments investigating the role of other YaeT complex genes in salmonella virulence are in progress.

ACKNOWLEDGEMENTS

This work was supported by the Science and Technology Assistance Agency under contract number APVT-51-012004, VEGA grant 2/6010/26 from the Slovak Academy of Sciences, and Wellcome Trust grant 065027/Z/01/Z. C. L. was funded by grant 069099/Z/02/A from the Wellcome Trust.

REFERENCES

- Alba, B. M. & Gross, C. A. (2004). Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol Microbiol* **52**, 613–619.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. O., Seidman, J. S., Smith, J. A. & Struhl, K. (1995). *Current Protocols in Molecular Biology*. Hoboken, NJ: Wiley.
- Beuzon, C. R. & Holden, D. W. (2001). Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions *in vivo*. *Microbes Infect* **3**, 1345–1352.
- Charlson, E. S., Werner, J. N. & Misra, R. (2006). Differential effects of *yfgL* mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. *J Bacteriol* **188**, 7186–7194.
- Crouch, M. L., Becker, L. A., Bang, I. S., Tanabe, H., Ouellette, A. J. & Fang, F. C. (2005). The alternative sigma factor σ^E is required for resistance of *Salmonella enterica* serovar Typhimurium to antimicrobial peptides. *Mol Microbiol* **56**, 789–799.
- Dartigalongue, C., Missiakas, D. & Raina, S. (2001). Characterization of the *Escherichia coli* sigma E regulon. *J Biol Chem* **276**, 20866–20875.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Eisinghorst, E. A. (1994). Measurement of invasion by gentamicin resistance. In *Methods in Enzymology*, vol. 236, pp. 405–420. New York: Academic Press.
- Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* **47**, 103–118.
- Fardini, Y., Chettab, K., Grepinet, O., Rochereau, S., Trotereau, J., Harvey, P., Amy, M., Bottreau, E., Bumstead, N. & other authors (2007). The YfgL lipoprotein is essential for type III secretion system expression and virulence of *Salmonella enterica* serovar Enteritidis. *Infect Immun* **75**, 358–370.
- Gentle, I. E., Burri, L. & Lithgow, T. (2005). Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* **58**, 1216–1225.
- Hoiseth, S. K. & Stocker, B. A. (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, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. *Infect Immun* **67**, 1560–1568.
- Humphreys, S., Rowley, G., Stevenson, A., Kenyon, W. J., Spector, M. P. & Roberts, M. (2003). Role of periplasmic peptidylprolyl isomerases in *Salmonella enterica* serovar Typhimurium virulence. *Infect Immun* **71**, 5386–5388.
- Kabir, M. S., Yamashita, D., Koyama, S., Oshima, T., Kurokawa, K., Maeda, M., Tsunedomi, R., Murata, M., Wada, C. & other authors (2005). Cell lysis directed by σ^E in early stationary phase and effect of induction of the *rpoE* gene on global gene expression in *Escherichia coli*. *Microbiology* **151**, 2721–2735.
- Kenyon, W. J., Sayers, D. G., Humphreys, S., Roberts, M. & Spector, M. P. (2002). The starvation-stress response of *Salmonella enterica* serovar Typhimurium requires σ^E , but not CpxR-regulated extracytoplasmic functions. *Microbiology* **148**, 113–122.
- Kormanec, J. (2001). Analyzing the developmental expression of sigma factors with S1-nuclease mapping. In *Nuclease Methods and Protocols*, pp. 481–494. Edited by C. H. Chein. Totowa, NJ: Humana Press.
- Linn, T. & St Pierre, R. (1990). Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. *J Bacteriol* **172**, 1077–1084.
- Maxam, A. M. & Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* **65**, 499–560.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Miticka, H., Rowley, G., Rezuchova, B., Homerova, D., Humphreys, S., Farn, J., Roberts, M. & Kormanec, J. (2003). Transcriptional analysis of the *rpoE* gene encoding extracytoplasmic stress response sigma factor sigma E in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* **226**, 307–314.
- Monack, D. M., Hersh, D., Ghorri, N., Bouley, D., Zychlinsky, A. & Falkow, S. (2000). *Salmonella* exploits caspase-1 to colonize Peyer's patches in a murine typhoid model. *J Exp Med* **192**, 249–258.
- Narita, S., Matsuyama, S. & Tokuda, H. (2004). Lipoprotein trafficking in *Escherichia coli*. *Arch Microbiol* **182**, 1–6.
- Ochsner, U. A., Vasil, A. I., Johnson, Z. & Vasil, M. L. (1999). *Pseudomonas aeruginosa* *fur* overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. *J Bacteriol* **181**, 1099–1109.
- Onufryk, C., Crouch, M. L., Fang, F. C. & Gross, C. A. (2005). Characterization of six lipoproteins in the σ^E regulon. *J Bacteriol* **187**, 4552–4561.
- Pribnow, D. (1975). Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. *Proc Natl Acad Sci U S A* **72**, 784–788.
- Rezuchova, B. & Kormanec, J. (2001). A two-plasmid system for identification of promoters recognized by RNA polymerase containing extracytoplasmic stress response σ^E in *Escherichia coli*. *J Microbiol Methods* **45**, 103–111.
- Rezuchova, B., Miticka, H., Homerova, D., Roberts, M. & Kormanec, J. (2003). New members of the *Escherichia coli* sigmaE regulon identified by a two-plasmid system. *FEMS Microbiol Lett* **225**, 1–7.
- Rhodijs, V. A., Suh, W. C., Nonaka, G., West, J. & Gross, C. A. (2005). Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4**, 1, e2.
- Rollenhagen, C., Sorensen, M., Rizos, K., Hurvitz, R. & Bumann, D. (2004). Antigen selection based on expression levels during infection facilitates vaccine development for an intracellular pathogen. *Proc Natl Acad Sci U S A* **101**, 8739–8744.
- Rowley, G., Stevenson, A., Kormanec, J. & Roberts, M. (2005). Effect of inactivation of *degS* on *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo*. *Infect Immun* **73**, 459–463.
- Rowley, G., Spector, M., Kormanec, J. & Roberts, M. (2006). Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol* **4**, 383–394.
- Sklar, J. G., Wu, T., Gronenberg, L. S., Malinverni, J. C., Kahne, D. & Silhavy, T. J. (2007). Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. *Proc Natl Acad Sci U S A* **104**, 6400–6405.
- Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Lewis, C., Roberts, M. & Kormanec, J. (2006). Identification of the σ^E regulon of *Salmonella enterica* serovar Typhimurium. *Microbiology* **152**, 1347–1359.
- Sydenham, M., Douce, G., Bowe, F., Ahmed, S., Chatfield, S. & Dougan, G. (2000). *Salmonella enterica* serovar Typhimurium *surA* mutants are attenuated and effective live oral vaccines. *Infect Immun* **68**, 1109–1115.
- Testerman, T. L., Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Libby, S. J. & Fang, F. C. (2002). The alternative sigma factor σ^E controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. *Mol Microbiol* **43**, 771–782.
- Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J. & Kahne, D. (2005). Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* **121**, 235–245.

Edited by: D. L. Gally