The periplasmic chaperone Skp is required for successful *Salmonella* Typhimurium infection in a murine typhoid model

Gary Rowley,¹ Henrieta Skovierova,² Andrew Stevenson,³ Bronislava Rezuchova,² Dagmar Homerova,² Claire Lewis,³ Aileen Sherry,³ Jan Kormanec² and Mark Roberts³

Correspondence

Gary Rowley
g.rowley@uea.ac.uk
Mark Roberts
m.roberts@vet.gla.ac.uk

¹School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

²Institute of Molecular Biology, Slovak Academy of Sciences, Dubravská cesta 21, 845 51 Bratislava, Slovak Republik

³Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK

The alternative sigma factor σ^E (*rpoE*) is essential for survival *in vivo* of *Salmonella* Typhimurium but is dispensable during growth in the laboratory. We have been identifying σ^E -regulated genes and studying their regulation and function to elucidate their potential role in the severe attenuation of *S. Typhimurium* *rpoE* mutants. In this study we identify five promoters that control the *rseP*, *yaeT* (*bamA*), *skp* region. A confirmed σ^E -dependent promoter, *yaeTp1*, and a second downstream promoter, *yaeTp2*, are located within the upstream gene *rseP* and direct expression of the downstream genes. The only known function of RseP is σ^E activation, and it is therefore not expected to be essential for *S. Typhimurium* *in vitro*. However, it proved impossible to delete the entire *rseP* gene due to the presence of internal promoters that regulate the essential gene *yaeT*. We could inactivate *rseP* by deleting the first third of the gene, leaving the *yaeT* promoters intact. Like the *rpoE* mutant, the *rseP* mutant exhibited severe attenuation *in vivo*. We were able to delete the entire coding sequence of *skp*, which encodes a periplasmic chaperone involved in targeting misfolded outer-membrane proteins to the β -barrel assembly machinery. The *skp* mutant was attenuated in mice after oral and parenteral infection. Virulence could be complemented by providing *skp* *in trans* but only by linking it to a heterologous σ^E -regulated promoter. The reason the *skp* mutant is attenuated is currently enigmatic, but we know it is not through increased sensitivity to a variety of RpoE-activating host stresses, such as H₂O₂, polymyxin B and high temperature, or through altered secretion of effector proteins by either the *Salmonella* pathogenicity island (SPI)-1 or the SPI-2 type III secretion system.

Received 7 October 2010
Revised 1 December 2010
Accepted 7 December 2010

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is capable of infecting a wide range of both warm- and cold-blooded hosts. A major limiting factor in the success of such a bacterial pathogen is its capacity to restore or negate damage caused by host defence mechanisms. The outer membrane of bacterial pathogens provides a critical first line of defence against a range of host innate defence mechanisms, including antimicrobial peptides and reactive oxygen

species, and therefore its maintenance is crucial. *S. Typhimurium* employs a number of stress response mechanisms that facilitate both outer membrane and periplasmic homeostasis during survival in harsh environments including, but not restricted to, infection, with critical contributions from the envelope stress response pathways (Rowley *et al.*, 2006).

The envelope stress response of *S. Typhimurium* and related bacterial species includes at least five partially overlapping pathways which are regulated by the alternative sigma factor σ^E (RpoE), the two-component systems CpxRA and BaeSR, the phage shock response and the Rcs phosphorelay system (Rowley *et al.*, 2006). In laboratory strains of *Escherichia coli*, σ^E is essential for cell viability (De Las Peñas *et al.*, 1997). In contrast, in *S. Typhimurium*

Abbreviations: BAM, β -barrel assembly machinery; CDS, coding sequence; CI, competitive index; FRT, flippase recognition target; IP, intraperitoneal(ly); OMP, outer-membrane protein; SPI, *Salmonella* pathogenicity island; TSP, transcriptional start point; T3SS, type III secretion system; UTI, urinary tract infection; WT, wild-type.

and other pathogens, σ^E is not essential for cell viability in the laboratory but is critical for survival in a mammalian host (Craig *et al.*, 2002; Humphreys *et al.*, 1999; Kovacicova & Skorupski, 2002). Maximal activation of the σ^E response is dependent on proteolysis of the cognate anti-sigma factor RseA (Alba *et al.*, 2002), an event that requires two inner-membrane proteases, DegS and RseP (YaeL, EcfE). DegS is a member of the HtrA (DegP, Protease Do) serine protease family (Pallen & Wren, 1997). In the absence of an inducing signal, DegS is completely inactive. Activation of DegS protease activity requires the C-termini of misfolded outer-membrane proteins (OMPs) to bind to the DegS PDZ domain (Walsh *et al.*, 2003). This results in a conformational change to DegS and exposure of the protease domain. The DegS protease domain cleaves the periplasmic domain of RseA (Alba & Gross, 2004; Alba *et al.*, 2002). Cleavage of RseA at the cytoplasmic face is performed by regulated intramembrane proteolysis (RIP) via the Zn-dependent metalloprotease RseP (Akiyama *et al.*, 2004; Kanehara *et al.*, 2002, 2003). Loss of DegS in *S. Typhimurium* results in phenotypes similar to those displayed by an *S. Typhimurium* σ^E mutant, although not with the same severity (Rowley *et al.*, 2005). RseP is essential for cell viability in *E. coli* (Dartigalongue *et al.*, 2001a) but its function in activation of σ^E should be dispensable for *S. Typhimurium* *in vitro*. However, like DegS and RpoE, we can expect that inactivation of RseP would lead to severe impairment of *S. Typhimurium* during infection. Release of σ^E from RseA allows transcriptional activation of σ^E -dependent genes, many of which have specific functions related to outer membrane or periplasmic homeostasis.

The σ^E regulon includes all members of the β -barrel assembly machinery (BAM) complex, which is required for insertion of β -barrel proteins into the outer membrane (for a review, see Tokuda, 2009). The BAM complex consists of the OMP BamA (YaeT), which is essential, and four lipoproteins, BamB (YfgL), BamC (NlpB), BamD (YfiO) and BamE (SmpA), none of which is essential. Several studies have elucidated roles for a number of the BAM complex components with regard to both the *S. Typhimurium* envelope stress response and the infection process (Amy *et al.*, 2004; Fardini *et al.*, 2007, 2009; Lewis *et al.*, 2008), consistent with the importance of maintaining outer membrane homeostasis for a successful infection.

Although not part of the BAM complex themselves, the periplasmic chaperones Skp, HtrA (DegP) and SurA are part of the OMP targeting pathway, and function to deliver OMPs to the BAM complex (Sklar *et al.*, 2007). SurA is the major chaperone required for transport of periplasmic OMPs to the BAM complex, whilst HtrA (DegP) and Skp appear to have more minor roles in OMP biogenesis under *in vitro* conditions, at least in *E. coli*, and function more to rescue OMPs which fall off the SurA pathway (Sklar *et al.*, 2007). SurA is required for *S. Typhimurium* to adhere to and invade eukaryotic cells and cause infection in a mouse model (Sydenham *et al.*, 2000). Although HtrA has a more

minor role in delivery of OMPs to BAM, it is required for many pathogens to cause a successful infection, with *S. Typhimurium* *htrA* mutants being defective for intracellular survival and attenuated in mice (Chatfield *et al.*, 1992; Johnson *et al.*, 1991). HtrA is a dual function protein that has both chaperone and protease activities, and at least in *S. Typhimurium* the protease activity is more important for virulence than the chaperone activity (Lewis *et al.*, 2009).

Surprisingly, compared with HtrA and SurA, information is relatively scarce regarding both the regulation of Skp expression and its importance during infection. In several global screens searching for σ^E regulon members, including ours, Skp has been identified as σ^E -regulated (Dartigalongue *et al.*, 2001b; Skovierova *et al.*, 2006), whilst loss of Skp also results in induction of the σ^E response (Missiakas *et al.*, 1996). In an *E. coli* urinary tract infection (UTI) model, loss of *skp* results in a reduction of virulence (Redford & Welch, 2006), although this phenotype cannot be restored by *skp in trans*.

In this present study we performed transcriptional mapping with S1 nuclease to identify the regulatory regions of the *S. Typhimurium* *rseP*, *yaeT* (*bamA*) and *skp* genes. We also constructed *skp* and *rseP* mutants and investigated their phenotypes *in vitro* and *in vivo*.

METHODS

Bacterial strains, plasmids and culture conditions. All strains and plasmids used in this study are listed in Table 1. Unless stated, bacteria were grown in Luria-Bertani (LB) broth and supplemented with 1.5 % agar to form solid media. When required, the media were supplemented with 100 μ g ampicillin ml^{-1} , 40 μ g chloramphenicol ml^{-1} , 50 μ g kanamycin ml^{-1} or 50 μ g gentamicin ml^{-1} . In oxidative stress experiments, hydrogen peroxide was used at a final concentration of 0.002 % (v/v).

Isolation of RNA and S1-nuclease mapping. RNA was isolated and S1 mapping was carried out as previously described (Miticka *et al.*, 2003). Briefly, an overnight culture was diluted 500-fold into fresh LB and incubated at 37 °C with aeration to the exponential (OD_{600} 0.5) and stationary phases (OD_{600} 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 (negative control), respectively, was grown in LB with chloramphenicol to early exponential phase (OD_{600} 0.24) and expression of *rpoE* was then induced for 3 h with 0.2 % arabinose.

At the appropriate time point, the *S. Typhimurium* culture was chilled and washed with diethylpyrocarbonate (DEPC)-treated ice-cold 0.15 M NaCl. Total RNA was prepared and high-resolution S1 nuclease mapping performed. RNA samples (40 μ g) were hybridized to 0.02 pmol of the appropriate DNA probe labelled at the 5' end with [γ -³²P]ATP and treated with 120 U S1 nuclease (Promega). The probes used for S1-nuclease mapping were prepared by PCR amplification from *S. Typhimurium* SL1344 chromosomal DNA and the appropriate oligonucleotide (Table 2): probe 1 was a 375 bp DNA fragment prepared using the 5' end-labelled reverse primer *skp*STREV and the direct unlabelled primer *skp*STDIR; probe 2 was an 892 bp DNA fragment prepared using the 5' end-labelled reverse

Table 1. Strains and plasmids used in this studyAbbreviations: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
SL1344	<i>his</i> , mouse-virulent	Hoiseth & Stocker (1981)
GVB311	SL1344 <i>rpoE</i> ::Km ^R	Humphreys <i>et al.</i> (1999)
GVB1367	SL1344 <i>skp</i> ::Km ^R	This study
GVB2225	GVB1367 + pBBR1MCS-5 <i>htrA/skp</i>	This study
<i>rseP</i> -T	SL1344 <i>rseP</i> ::Km ^R	This study
<i>rseP</i> -T + <i>prseP</i> -1	SL1344 <i>rseP</i> ::Km ^R + <i>prseP</i> -1	This study
Plasmids		
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 _{<i>araB</i>} and <i>γ</i> , <i>β</i> , <i>exo</i> genes	Datsenko & Wanner (2000)
pBBR1MCS-5	Gm ^r , broad-host-range vector; P _{<i>lac</i>}	Kovach <i>et al.</i> (1995)
pBBR1MCS-5 <i>htrA/skp</i>	pBBR1MCS-5 containing <i>S. Typhimurium</i> <i>skp</i> under the control of the <i>htrA</i> promoter region	This study
pAC7	Cm ^R , low-copy-number expression vector with P _{BAD} promoter	Rezuchova & Kormanec (2001)
pAC- <i>rpoEST</i> 4	Cm ^R , pAC7 containing <i>S. Typhimurium</i> <i>rpoE</i>	Skovierova <i>et al.</i> (2006)
<i>prpoEP</i> 3	Ap ^R , pTL61T containing <i>σ</i> ^E -dependent promoter	Miticka <i>et al.</i> (2003)
<i>prseP</i> -1	Cm ^R , pAC7 containing <i>S. Typhimurium</i> <i>rseP</i>	This study
pACYC-T7	Cm ^R , pACYC184 plus T7 promoter from pAR2156	Yasukawa <i>et al.</i> (1995)
pT- <i>rpoE</i>	Cm ^R , pACYC-T7 containing <i>S. Typhimurium</i> <i>rseP</i>	This study

primer *lpxDSTREV* and the direct unlabelled primer *skpSTDIR*; probe 3 was a 1068 bp DNA fragment prepared using the 5' end-labelled reverse primer *rsePREV* and the direct unlabelled primer *cdsADIR*; probe 4 was a 1242 bp DNA fragment prepared using the 5' end-

labelled reverse primer *yaeTREV* and the direct unlabelled primer *yaeT2*; probe 5 was a 1554 bp DNA fragment prepared using the 5' end-labelled reverse primer *rseP2* and the direct unlabelled primer *cdsADIR*. Oligonucleotides were 5'-labelled with ³²P [ICN, 4500 Ci

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')
<i>skpSTREV</i>	CCTGTTGGAACAGATTACCC
<i>skpSTDIR</i>	CCAGGCAACATCCGTATGTCC
<i>lpxDSTREV</i>	GGATTACCATATAACGTAATGTGG
<i>rsePREV</i>	GCTCAACTCGCACACCGCAGC
<i>cdsADIR</i>	GGTGCGCTGCATGCCTTTGCT
<i>yaeTREV</i>	CCAACGGCGACTCGCTGCAGG
<i>yaeT2</i>	CGCCTTCAATAACAAACGG
<i>rseP2</i>	CCCAGTGACGTAAATCAAGCG
<i>rsePD4</i>	CCTGTTGTTACTGGTGTTACGACGCTCTAACGGAAGGTTTTATGATTCCGGGGATCCGTCGACC
<i>rsePD1</i>	CCATCAACGGCTTTAAGTTCCGTACCAGGCGCAATTTGCGCCGTGTAGGCTGGAGCTGCTTC
<i>cdsADIR</i>	GGTGCGCTGCATGCCTTTGC
<i>rseP2</i>	CCCAGTGACGTAAATCAAGCG
<i>skpRedF</i>	TTTATTGTGAAAAAGTGGTTATTAGCTGCAGGTCTTGGTTTGGCGATGGTGTGTAGGCTGGAGCTGCTTC
<i>skpRedR</i>	CATTACTTATTTAACCTGTTTCAGTACGTCAGCGGTGATGTCTTTCACATCATATGAATATCCTCCTTAG
<i>skpExtF</i>	GGGGCCGTTGGTCTTCTCCTACG
<i>skpExtR</i>	GGATTACCATATAACGTA ATGTG
<i>htrAfwEcoRI</i>	CGGAATTCTAACTAAGTAAAAAGCTTGTGCGCTTAACGACTTTTCG CGA
<i>htrArv</i>	AAGACCTGCAGCTAATAACCACTTTTTCACGTGTTTCAATCTCGATTAAACAGGTAACGCAAAA
<i>skpfw</i>	TTTTGCGTTACCTGTTAATCGAGATTGAAACACGTGAAAAAGTGGTTATTAGCTGCAGGTCTT
<i>skprvNcoI</i>	CATGCCATGGTTATTTAACCTGTTTCAGTACGTCAGCGGTGATGT
<i>rsePFwNde</i>	CCCCCATATGCTGAGTATTCTCTGGAATCTGG
<i>rsePRvBam</i>	CCCCCGGATCCTAACTAACTCTCTTACAACC
<i>rpoESal</i>	CATGGTTCTTGTGCACTTTACTAAACA TGG
<i>rpoENde</i>	GCTCGCTCATATGAGGTAATGTC

mmol⁻¹ (166.5 TBq mmol⁻¹)] and T4 polynucleotide kinase (Promega). The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments.

Mutant construction. An *rseP* mutant was constructed in *S. Typhimurium* SL1344 using λ Red mutagenesis (Datsenko & Wanner, 2000). To decrease background, the template pKD4 plasmid was digested with *Hind*III and an agarose gel-purified 1635 bp DNA fragment was used for PCR with mutagenesis primers *rsePD4* and *rsePD1* to amplify a kanamycin-resistance (Km^R) gene cassette with flanking regions homologous to *rseP*. The amplified DNA fragment was column purified (Qiagen) and electroporated into *S. Typhimurium* SL1344pKD46. Mutation of *rseP* was confirmed by PCR using primers *cdsADir* and *rseP2*, which are external to the site of mutagenesis; as well as internal primers within the Km^R cassette, k1 and k2 (Datsenko & Wanner, 2000). P22 transduction was used to transfer the *rseP* mutation into a clean SL1344 background. The *S. Typhimurium* SL1344 *rseP* mutant strain was named *S. Typhimurium rseP-T*. The *S. Typhimurium* Δ skp::kan mutant (GVB1367) was also mutated by λ Red mutagenesis using primers *skpRedF* and *skpRedR* to amplify the DNA fragment for recombination from pKD4. The entire coding sequence (CDS) of *skp* was replaced by a flippase recognition target (FRT)-flanked Km^R cassette from template plasmid pKD4, and mutagenesis was confirmed with primers *skpExtF* and *skpExtR* flanking the site of deletion and the locus P22 transduced into a clean *S. Typhimurium* SL1344 background.

Construction of an Skp complementing plasmid. The *htrA* promoter region from *S. Typhimurium* SL1344 was amplified using primers *htrAfwEcoRI* and *htrArv*. The *skp* gene from *S. Typhimurium* SL1344 was amplified using primers *skpfw* and *skprvNcoI*. The resulting PCR products were purified, mixed in equal amounts and joined together using splicing by overlapping extension PCR with primers *htrAfwEcoRI* and *skprvNcoI*. The resulting fragment was then digested with *EcoRI* and *NcoI*. Plasmid pBBR1MCS-5 was similarly digested and the larger DNA fragment produced from this was gel-purified and ligated to the *skp* gene fragment described above. The resulting plasmid was transformed into *E. coli* TOP10 cells and selected on 50 μ g gentamicin ml⁻¹. Clones were verified by DNA sequencing. The resulting plasmid was transformed into SL1344 Δ skp (GVB1367) to produce strain GVB 2225.

Complementation of *S. Typhimurium rseP-T*. To complement the *rseP* mutation, *S. Typhimurium rseP-T* was electroporated with plasmid *prseP-1*. This contains *rseP* under the control of the three *S. Typhimurium rpoE* promoters and a ribosome-binding site. *prseP-1* was constructed by cloning a 1400 bp *rseP* fragment into plasmid pT-*rpoE*. Plasmid pT-*rpoE* was prepared as follows. A 430 bp DNA fragment containing the *S. Typhimurium* SL1344 *rpoE* promoter region including the ribosome-binding site was amplified from chromosomal DNA of *S. Typhimurium* SL1344 by PCR using primers *rpoESal* and *rpoENde*, introducing *SalI* and *NdeI* at the beginning and the end of the promoter region. The PCR fragment was digested with *SalI* and *NdeI* and cloned into pACYC-T7 (Yasukawa *et al.*, 1995), resulting in pT-*rpoE*. The *rseP* insert was prepared by PCR amplification using *S. Typhimurium* SL1344 chromosomal DNA as template and primers *rsePFwNde* and *rsePRvBam*, which incorporated an *NdeI* site in the *rseP* translation initiation codon and a *BamHI* site just downstream of the stop codon. All the DNA fragments were amplified by proof-reading *Pfu* DNA polymerase (Stratagene) and verified by nucleotide sequencing.

Preparation of secreted proteins. Bacteria were pre-cultured in LB for 8 h at 37 °C, washed twice, and inoculated at 1:50 dilution in LB [*Salmonella* pathogenicity island (SPI)-1-inducing conditions] or minimal medium (SPI-2-inducing conditions). The composition of

minimal medium has been described elsewhere (Nikolaus *et al.*, 2001). Overnight cultures were adjusted to equal amounts of bacteria by OD₆₀₀. Proteins in the supernatant fractions were recovered by precipitation with TCA (10 %, v/v, final concentration) at 4 °C overnight and centrifugation for 15 min at 10 000 g. The pellet was washed twice with 50 mM Tris-buffered ethanol and recovered by centrifugation at 10 000 g for 15 min. The final pellet was air-dried and resuspended in SDS-PAGE sample buffer (Sigma). Proteins secreted by the type III secretion system (T3SS) of SPI-2 were recovered from the bacterial surface as described before (Nikolaus *et al.*, 2001).

Western blotting. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen) by electroblotting. Proteins were visualized by incubating the membranes with polyclonal antisera or monoclonal antibodies specific for SipC or SseB (kindly donated by Professor Michael Hensel, Mikrobiologisches Institut, Universitätsklinikum Erlangen, Erlangen, Germany), followed by the appropriate anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate (Dako) and 4-chloro-1-naphthol substrate (Sigma).

Murine infections. For all *in vivo* studies, strains were grown statically overnight at 37 °C, centrifuged, washed, resuspended to the appropriate concentration in sterile PBS, and administered to mice in doses of 200 μ l. Female BALB/c mice (6–8 weeks old, Harlan) were used throughout. Initial assessment of Δ skp virulence was carried out by using the well-described competitive index (CI) assay (Beuzón & Holden, 2001), where ~1000 c.f.u. each of the parent strain and isogenic mutant was administered in a 1:1 ratio via the intraperitoneal (IP) route and c.f.u./organ determined at 72 h post-infection. For oral infection, the inoculum [wild-type (WT), ~5 $\times$ 10⁵ c.f.u.; *skp* mutant, ~5 $\times$ 10⁶ c.f.u.] was administered via oral gavage, and mice were culled 5 days later. Organs (livers, spleens, Peyer's patches and mesenteric lymph nodes) were isolated and homogenized, and numbers of bacteria present were enumerated by viable counting. The attenuation of the *rseP* mutant was compared with that of the *rpoE* and *degS* mutants by inoculating groups of five mice intraperitoneally with one of the mutants and then the c.f.u. in the spleens was determined 24 h post-infection. The severe attenuation of *S. Typhimurium* lacking fully functional σ^E meant that we had to assay for c.f.u. per organ at 24 h post IP infection as opposed to the usual 72 h for this route of infection. At 72 h, no bacteria remained in the spleens and livers of BALB/c mice. Statistical significance was determined ($P < 0.05$) using a non-parametric ANOVA (Kruskal–Wallis).

RESULTS

Transcriptional mapping of the σ^E -dependent BamA region

Using the optimized *E. coli* two-plasmid system for the identification of promoters recognized by *S. Typhimurium* σ^E , we had previously identified 34 σ^E -dependent promoters directing expression of 62 genes (Skovierova *et al.*, 2006). One of these σ^E -dependent promoters was located in the *rseP* coding region, and we hypothesized that this would direct expression of the downstream genes *yaeT* (*bamA*), *skp*, *lpxD* and *fabZ*.

To characterize this σ^E -dependent promoter and expression of these genes we employed high-resolution S1-nuclease mapping using five 5'-labelled probes (Fig. 1a) and RNA

Fig. 1. Transcriptional analysis of the *cdsA*, *rseP*, *yaeT*, *skp* and *lpxD* region in *S. Typhimurium*. (a) Scheme of the region in *S. Typhimurium*. The positions of the identified promoters are indicated by bent arrows. The lines below the map represent DNA fragments (5'-labelled at the end marked with an asterisk) that were used as probes in S1-nuclease mapping experiments. (b) High-resolution S1-nuclease mapping of the TSPs for the *S. Typhimurium* promoters directing expression of the *rseP*, *yaeT*, *skp* region. The corresponding 5'-labelled DNA probes were hybridized in parallel with 40 µg RNA and treated with 120 U S1 nuclease. 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. RNA from *S. Typhimurium* SL1344 containing pAC-*rpoEST4* (+) or pAC7 (-) was grown to early exponential phase and induced with 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. All S1-nuclease mapping experiments were performed twice using independent sets of RNA with similar results.

isolated from *S. Typhimurium* SL1344 and its isogenic *rpoE* mutant GVB311 (Miticka *et al.*, 2003). Using all of the labelled probes, only five RNA-protected fragments were identified, which correspond to *rsePp*, *yaeTp1*, *yaeTp2*, *skpp1* and *skpp2* promoters (Figs 1b and 2). Interestingly all the promoters were located within genes. No RNA-protected fragments were identified with tRNA as a control (Fig. 1b, lane C). Only one promoter, *yaeTp1*, was clearly σ^E -dependent, based on the presence of an RNA-protected fragment corresponding to *yaeTp1* in WT *S. Typhimurium*

which was absent in the *rpoE* mutant grown under identical conditions (Fig. 1b). The σ^E -dependent *yaeTp1* promoter was induced at stationary phase and by cold shock, conditions known to induce σ^E -dependent promoters in *S. Typhimurium* (Miticka *et al.*, 2003). Transcription from *yaeTp1* was also induced by overexpression of *rpoE* from pAC-*rpoEST4* (which has the *S. Typhimurium* *rpoE* gene under the control of the arabinose-inducible P_{BAD} promoter) but not the negative control strain *S. Typhimurium* SL1344 pAC7. The transcriptional start point (TSP) of

yaeT is in an identical position to that identified previously using the two-plasmid system (Skovierova *et al.*, 2006). These results clearly indicate that the *S. Typhimurium yaeT* promoter is dependent upon σ^E *in vivo*. The sequence of the promoter (Fig. 2) is highly similar to the σ^E -consensus sequence, GGAAGTT-N₁₅-GTCTAA (Skovierova *et al.*, 2006).

In addition to the single σ^E -dependent *yaeT* promoter, there are four other σ^E -independent promoters. Based on their sequences (Fig. 2), they would all appear to bind RNA polymerase containing the principal sigma factor σ^{70} , as they are similar to its consensus sequence TTGACA-N₁₆₋₁₈-TATAAT (Pribnow, 1975). Both *skp* promoters displayed similar activity in the exponential phase and under

Fig. 2. Nucleotide sequences of the *S. Typhimurium rseP*, *yaeT* and *skp* promoter regions. The deduced protein products corresponding to the *cdsA*, *rseP*, *yaeT* and *skp* genes are given as the single-letter amino acid codes under 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, underlined type. The numbers correspond to the nucleotide positions, which refer to the sequence with GenBank/EMBL/DBJ accession number AE006468.

cold-shock conditions, and their activity was dramatically decreased after heat shock and at stationary phase (Fig. 1b).

Based on the strong end-to-end signal between probe 1 and RNA, it is likely that in addition to *yaeT* the σ^E -dependent *yaeTp1* promoter also directs expression of downstream genes including *skp*. Transcriptional profiling with *Salmonella* microarrays using RNA from *S. Typhimurium* SL1344 overexpressing *rpoE* from pAC-*rpoEST4* versus control plasmid pAC7 revealed ratio values that confirm this suggestion for *yaeT* (5.78) and *skp* (7.13) (G. Rowley, unpublished results). This is in agreement with published transcriptomic data for an *S. Typhimurium* *rpoE* mutant during stationary phase, in which *yaeT* and *skp* are downregulated in the absence of *rpoE* (Bang *et al.*, 2005).

Construction of *S. Typhimurium* *skp* and *rseP* mutants

We attempted to delete the *skp* and *rseP* genes from the *S. Typhimurium* SL1344 chromosome using λ Red mutagenesis (Datsenko & Wanner, 2000). We had no difficulty in replacing the *skp* gene with a Km^R cassette to create strain GVB1367. However, in contrast, despite repeated attempts, we were unable to delete the whole *rseP* CDS. Although RseP is essential in *E. coli* (Kanehara *et al.*, 2001), we did not expect this to be the case in *S. Typhimurium* as both *rpoE* and *degS* mutants are viable. The primary role of RseP is to perform the secondary cleavage event of σ^E from RseA for full activation of the σ^E pathway, which we know is not essential for *S. Typhimurium* viability (Humphreys *et al.*, 1999).

From our transcriptional studies, the σ^E -dependent and independent promoters *yaeTp1* and *yaeTp2* that direct expression of the *yaeT* gene are located in the *rseP* (*yaeL*) CDS (Fig. 2). *YaeT* (BamA) is essential in *S. Typhimurium*, as we were only able to disrupt the chromosomal copy of *yaeT* in the presence of *yaeT* *in trans* (J. Kormanec, unpublished results). We predicted that the inability to delete the whole of *rseP* was due to the parallel deletion of the *yaeTp1* and *yaeTp2* promoters that may be crucial for the expression of *yaeT*. This appears to be the case. We were able to inactivate *rseP* (*rseP*-T) by deleting the first 426 bases of the *rseP* sequence, which retained the *yaeTp1* and *yaeTp2* promoters.

RseP is required for σ^E activation and *S. Typhimurium* infection of mice

rseP-T had no stress-induced σ^E activity as determined by S1 mapping of the σ^E -dependent *rpoEP3* promoter. Loss of σ^E in *Salmonella* results, amongst other phenotypes, in loss of virulence in a murine typhoid model. This makes a good phenotypic check for deregulation of σ^E activity. The c.f.u. in the spleen of mice infected with the *degS* mutant is significantly different from that of the *rpoE* mutant, indicating DegS-independent RpoE activation (Rowley *et al.*, 2005). An *rseP* mutant was severely impaired in its

ability to survive and replicate in the mouse model post IP infection (Fig. 3a), consistent with loss of σ^E activity, and the c.f.u. in the spleen at 24 h post-infection was not statistically different from that for mice infected with the *rpoE* mutant.

Characterization of an *S. Typhimurium* *skp* mutant

As *Skp* is a member of the RpoE regulon we first assayed whether the *skp* mutant has any of the well-recognized phenotypes associated with loss of σ^E *in vitro*, caused by stress which damages the bacterial envelope. We could find no difference in growth curves between the *skp* mutant and the isogenic parent strain when cultured at high temperature (46 °C), or significant differences in zones of inhibition on disc diffusion assays in the presence of the antimicrobial peptide polymyxin B or under oxidative

Fig. 3. Systemic and oral infection of BALB/c mice. (a) Single doses of $\sim 1 \times 10^5$ c.f.u. of the *rseP*, *degS* or *rpoE* mutant were given individually via the IP route to groups of four to five female BALB/c mice. After 24 h, bacteria in the spleens were enumerated. The bars show mean values; error bars, SD. (b) Mice were inoculated orally with either 5×10^5 c.f.u. of the WT strain or $\sim 5 \times 10^6$ c.f.u. of the *skp* mutant, and the number of bacteria present in different organs was determined 5 days later. Bars show mean results for five mice; error bars, SD. MLN, mesenteric lymph node; PP, Peyer's patches.

stress generated by hydrogen peroxide (data not shown). To ascertain any involvement of Skp in *Salmonella* infection, a competition assay was performed with the *skp* mutant GVB1367 versus its isogenic parent (WT) strain in BALB/c mice. A dose containing equal numbers of the *skp* and WT strains (2×10^3 c.f.u. per strain) was inoculated IP into mice, and c.f.u. in livers and spleens enumerated 3 days later. The CI was 0.038 ($P < 0.05$) and clearly demonstrated that the *skp* mutant was significantly attenuated. This loss of virulence was restored by the presence of *skp*, *in trans*, with GVB2225 (the *skp*-complemented strain) versus WT producing a CI of 0.59, which is not significantly attenuated ($P > 0.05$). Fig. 3(b) depicts the organ load in the spleen, liver, mesenteric lymph nodes and Peyer's patches following oral infection with either the isogenic parent strain or GVB1367. In all cases, except for the Peyer's patches, the organ load was significantly ($P < 0.05$) reduced in mice infected with the *skp* mutant compared with the parent strain. Although Skp has only a minor role under *in vitro* conditions, it does play a significant role during *S. Typhimurium* infection of mice.

Loss of Skp does not affect SPI-1 or SPI-2 type III secretion

A link between the BAM complex and the SPI-1 type T3SS has been reported before, with loss of BamB or BamD resulting in reduced secretion of SPI-1 effector proteins (Fardini *et al.*, 2007, 2009).

As Skp is a BAM-associated chaperone we determined whether the virulence phenotype observed with the *S. Typhimurium* *skp* mutant arises through disturbance of protein secretion via either the SPI-1 or the SPI-2 T3SS. We analysed secretion of the SPI-1 effector protein SipC and the SPI-2 secreted protein SseB under SPI-1- and SPI-2-inducing conditions, respectively (Fig. 4). Similar to the findings reported for SurA (Fardini *et al.*, 2009), we saw no significant difference in the secretion of these proteins in the absence of Skp compared with the isogenic parent strain under these *in vitro* inducing conditions. The

virulence phenotype of the *skp* mutant is therefore unlikely to be due to a defect in one of the T3SSs.

RpoE has been shown to regulate a subset of SsrB-regulated genes, with loss of RpoE resulting in a decrease in secretion of SseB (Osborne & Coombes, 2009). As a positive control for our *skp* analysis, and in agreement with Osborne & Coombes (2009), we saw a marked reduction in secretion of SseB in the absence of either RpoE or RseP compared with the isogenic parent. The role of RpoE in regulating type III secretion expression appears to be limited to SPI-2, as secretion of the SPI-1 effector protein SipC was not significantly different in either the RseP or the RpoE mutant.

DISCUSSION

Members of the RpoE regulon in enterobacteria are generally proteins that function to maintain the outer membrane (for a review, see Rowley *et al.*, 2006). We are currently investigating the contribution of various RpoE regulon members to the severe phenotypes seen in an *S. Typhimurium* *rpoE* deletion mutant, including sensitivity to antimicrobial peptides, reduced defence against oxidative stress and stationary phase survival, and severe attenuation in the murine typhoid model. One identified member of the *S. Typhimurium* RpoE regulon is Skp (HlpA, OmpH), which forms part of the *yaeTrsePskp* region.

Skp (a 17 kDa protein) is a periplasmic chaperone, and is synthesized as a precursor with an N-terminal signal sequence (Thome & Müller, 1991). Skp was named by Kleppe and co-workers (Hoick & Kleppe, 1988), who identified the *E. coli* gene, although the protein was first purified in 1979 as an LPS-associated protein of *Salmonella minnesota* (Geyer *et al.*, 1979). Skp was also purified on the basis that it was the predominant protein bound to unfolded OmpF in an affinity chromatography assay (Chen & Henning, 1996). Skp participates in the early folding events of OMPs such as OmpA (De Cock *et al.*, 1999) and binds an N-terminal region of OmpA immediately after folding begins (Schäfer *et al.*, 1999). The crystal structure of Skp has also been solved by two groups (Korndörfer *et al.*, 2004; Walton & Sousa, 2004). It forms a stable homotrimer in solution (Schlapschy *et al.*, 2004), and the protein has an overall basket-like shape. A comparison of the relative sizes of OmpA and Skp suggests that OmpA is bound in a compacted state, as the unfolded state would be too large to be accommodated by Skp and would therefore be able to interact with other polypeptides (Korndörfer *et al.*, 2004). *E. coli* Skp has also been identified as a chemoattractant of monocytes and polymorphonuclear leukocytes via the C5a receptor, one of the most important leukocyte receptors involved in the inflammatory reaction (Shrestha *et al.*, 2004). As Skp is widely distributed in Gram-negative bacteria, those authors suggest that recognition of Skp by leukocytes could be of

Fig. 4. Analysis of SPI-1 and SPI-2 type III secretion in the absence of *skp* and *rseP*. Bacterial strains were grown under SPI-1- or SPI-2-inducing conditions and secreted or surface-attached fractions were prepared as described above. The presence of SipC or SseB was detected by Western blotting using specific antisera against these proteins.

benefit to the host innate defence against Gram-negative bacteria (Shrestha *et al.*, 2004). In laboratory strains of *E. coli*, Skp is not essential for cell viability (Rizzitello *et al.*, 2001), but loss of Skp does lead to a moderate reduction in proteins in the outer membrane (Chen & Henning, 1996). As well as being regulated by σ^E itself, loss of functional Skp has also been shown to induce the σ^E regulon (Missiakas *et al.*, 1996).

In the current study we have shown that regulation of the *rsePskpyaeT* region in *S. Typhimurium* appears to be complex. Expression of these genes is governed by at least five promoters, only one of which, *yaeTp1*, is σ^E -dependent. It is likely that *yaeTp1*, as well as regulating expression of *yaeT*, also controls the downstream genes, including *skp* (based on the strong end-to-end signal between probe 1 and RNA). The sequence of this promoter is consistent with a consensus sequence for a σ^E -dependent promoter (Miticka *et al.*, 2003) and an equivalent promoter has been identified in *E. coli* (Rhodius *et al.*, 2006). Early work suggested that there were three σ^E -dependent promoters in this locus (Dartigalongue *et al.*, 2001b). However, none of these promoters fits the σ^E consensus sequence, and it appears after closer inspection that regulation of these genes in *E. coli* and *S. Typhimurium* is likely to be similar if not the same.

The absence of Skp did not affect the ability of *S. Typhimurium* to resist a variety of stresses. This would be consistent with Skp playing a minor role in OMP biogenesis *in vitro*. However, induction of the σ^E pathway in *E. coli* and *S. Typhimurium skp* mutants (Missiakas *et al.*, 1996; G. Rowley, unpublished results) indicates a disruption in cell envelope homeostasis and the periplasmic presence of unfolded OMPs in these mutants.

In contrast to the lack of effect of the absence of Skp in *S. Typhimurium* during growth under stress *in vitro*, inactivation of *skp* significantly attenuates *S. Typhimurium in vivo* during infection of mice. This indicates a specific role for Skp in the mammalian host environment. Skp appears to be particularly important during systemic infection and growth in organs such as the liver and spleen. The *skp* mutation did not appear to affect the ability of *S. Typhimurium* to colonize or grow in the Peyer's patches. This phenotype, a defect in growth at systemic but not at gut-associated sites, is also exhibited by *S. Typhimurium* strains with mutations in other σ^E -regulated genes such as *htrA* and *smpA* (Lewis *et al.*, 2008, 2009).

An *skp* mutant of a uropathogenic strain of *E. coli*, CFT073, is attenuated in a UTI mouse model, although this phenotype could not be complemented (Redford & Welch, 2006). Our results provide a possible reason for this. We show that there are no promoters in the intragenic region between *rseP* and *skp* and that all of the promoters that could control *skp* expression are present in the CDSs of upstream genes. The authors of the *E. coli* study do not provide information on the amount of upstream DNA

included in the constructs used to try to complement *skp* (Redford & Welch, 2006), but if they only included the region between the end of *rseP* and the start of *skp* then *skp* would not be expressed. Due to the regulatory complexity of *skp* identified in this current study we deliberately controlled the expression of *skp in trans* with the σ^E -dependent promoter of *HtrA*, which led to complete complementation of the *in vivo* defect.

The level of attenuation of a Δskp mutant, although significant in comparison with the WT strain, does not display the degree of attenuation of an *S. Typhimurium* $\Delta rpoE$ mutant. It is, however, a candidate for immunological studies as a potential carrier or vaccine strain, as although on the whole severely attenuated, it is still present in large numbers within the Peyer's patches, so one would imagine that it is capable of eliciting a protective mucosal response. We are currently investigating the potential of the *skp* mutant as a vaccine strain.

Whilst determining both the regulation of Skp and its role in *S. Typhimurium* pathogenesis, we also characterized the role of *rseP* in this process. RseP is well characterized as performing proteolytic cleavage of RseA at the cytoplasmic face of the inner membrane to liberate σ^E , normally after DegS has proteolytically cut RseA at the periplasmic side. We have previously demonstrated a role for DegS in *S. Typhimurium* pathogenesis, but showed that the attenuation is not as severe as that observed when *rpoE* is deleted, indicating that an alternative signalling pathway exists (Rowley *et al.*, 2005). A recent study has demonstrated that under specific acid-stress conditions, RseP is able to cleave RseA and activate the σ^E pathway independently of DegS (Muller *et al.*, 2009). Those authors provide evidence that RseP and σ^E , but not DegS, is required for acid adaptation of *Salmonella* to the macrophage environment, which accounts at least in part for the attenuation of an *rpoE* mutant. In support of this evidence we find that an *rseP* mutant of *S. Typhimurium*, as well as demonstrating some of the characteristic *rpoE* mutant-like phenotypes *in vitro*, is severely attenuated in the mouse typhoid model.

In conclusion, the *rsePyaeTskp* genetic locus is under complex regulation, and this complexity is reflected in the requirement for all of these genes for either *S. Typhimurium* viability in the case of *yaeT* (*bamA*) or pathogenesis in the case of *rseP* and *skp*. Understanding the other regulatory proteins involved in controlling transcription of these genes is likely to shed more light on the integration of the σ^E response with other regulatory circuits, which may also be required for *S. Typhimurium* pathogenesis.

ACKNOWLEDGEMENTS

We would like to thank Michael Hensel (see above) for his generous donation of antibodies. This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) responsive mode grant BB/G020582/1 (G.R.), BBSRC research grant BB/C508485/1 (M.R. and A.S.), Wellcome Trust studentship 069099/Z/02/A (C.L.),

the Science and Technology Assistance Agency under contract number APVT-51-012004, and VEGA grant 2/0104/09 from the Slovak Academy of Sciences (J. K.).

REFERENCES

- Akiyama, Y., Kanehara, K. & Ito, K. (2004). RseP (YaeL), an *Escherichia coli* RIP protease, cleaves transmembrane sequences. *EMBO J* **23**, 4434–4442. doi:10.1038/sj.emboj.7600449.
- Alba, B. M. & Gross, C. A. (2004). Regulation of the *Escherichia coli* σ^E -dependent envelope stress response. *Mol Microbiol* **52**, 613–619. doi:10.1111/j.1365-2958.2003.03982.x.
- Alba, B. M., Leeds, J. A., Onufryk, C., Lu, C. Z. & Gross, C. A. (2002). DegS and YaeL participate sequentially in the cleavage of RseA to activate the σ^E -dependent extracytoplasmic stress response. *Genes Dev* **16**, 2156–2168. doi:10.1101/gad.1008902.
- Amy, M., Velge, P., Senocq, D., Bottreau, E., Mompert, F. & Virlogeux-Payant, I. (2004). Identification of a new *Salmonella enterica* serovar Enteritidis locus involved in cell invasion and in the colonisation of chicks. *Res Microbiol* **155**, 543–552. doi:10.1016/j.resmic.2004.03.005.
- Bang, I. S., Frye, J. G., McClelland, M., Velayudhan, J. & Fang, F. C. (2005). Alternative sigma factor interactions in *Salmonella*: σ^E and σ^H promote antioxidant defences by enhancing σ^S levels. *Mol Microbiol* **56**, 811–823. doi:10.1111/j.1365-2958.2005.04580.x.
- Beuzón, 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. doi:10.1016/S1286-4579(01)01496-4.
- Chatfield, S. N., Strahan, K., Pickard, D., Charles, I. G., Hormaeche, C. E. & Dougan, G. (1992). Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. *Microb Pathog* **12**, 145–151. doi:10.1016/0882-4010(92)90117-7.
- Chen, R. & Henning, U. (1996). A periplasmic protein (Sklp) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Mol Microbiol* **19**, 1287–1294. doi:10.1111/j.1365-2958.1996.tb02473.x.
- Craig, J. E., Nobbs, A. & High, N. J. (2002). The extracytoplasmic sigma factor, final σ^E , is required for intracellular survival of nontypeable *Haemophilus influenzae* in J774 macrophages. *Infect Immun* **70**, 708–715. doi:10.1128/IAI.70.2.708-715.2002.
- Dartigalongue, C., Loferer, H. & Raina, S. (2001a). EcfE, a new essential inner membrane protease: its role in the regulation of heat shock response in *Escherichia coli*. *EMBO J* **20**, 5908–5918. doi:10.1093/emboj/20.21.5908.
- Dartigalongue, C., Missiakas, D. & Raina, S. (2001b). Characterization of the *Escherichia coli* σ^E regulon. *J Biol Chem* **276**, 20866–20875. doi:10.1074/jbc.M100464200.
- 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. doi:10.1073/pnas.120163297.
- De Cock, H., Schäfer, U., Potgeter, M., Demel, R., Müller, M. & Tommassen, J. (1999). Affinity of the periplasmic chaperone Sklp of *Escherichia coli* for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Sklp in the biogenesis of outer membrane protein. *Eur J Biochem* **259**, 96–103. doi:10.1046/j.1432-1327.1999.00010.x.
- De Las Peñas, A., Connolly, L. & Gross, C. A. (1997). σ^E is an essential sigma factor in *Escherichia coli*. *J Bacteriol* **179**, 6862–6864.
- Fardini, Y., Chettab, K., Grépinet, O., Rochereau, S., Trottereau, 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. doi:10.1128/IAI.00716-06.
- Fardini, Y., Trottereau, J., Bottreau, E., Souchard, C., Velge, P. & Virlogeux-Payant, I. (2009). Investigation of the role of the BAM complex and SurA chaperone in outer-membrane protein biogenesis and type III secretion system expression in *Salmonella*. *Microbiology* **155**, 1613–1622. doi:10.1099/mic.0.025155-0.
- Geyer, R., Galanos, C., Westphal, O. & Golecki, J. R. (1979). A lipopolysaccharide-binding cell-surface protein from *Salmonella minnesota*. Isolation, partial characterization and occurrence in different Enterobacteriaceae. *Eur J Biochem* **98**, 27–38. doi:10.1111/j.1432-1033.1979.tb13156.x.
- Hoick, A. & Kleppe, K. (1988). Cloning and sequencing of the gene for the DNA-binding 17K protein of *Escherichia coli*. *Gene* **67**, 117–124. doi:10.1016/0378-1119(88)90014-5.
- Hoise, S. K. & Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239. doi:10.1038/291238a0.
- Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A. B. & Roberts, M. (1999). The alternative sigma factor, σ^E , is critically important for the virulence of *Salmonella typhimurium*. *Infect Immun* **67**, 1560–1568.
- Johnson, K., Charles, I., Dougan, G., Pickard, D., O'Gaora, P., Costa, G., Ali, T., Miller, I. & Hormaeche, C. (1991). The role of a stress-response protein in *Salmonella typhimurium* virulence. *Mol Microbiol* **5**, 401–407. doi:10.1111/j.1365-2958.1991.tb02122.x.
- Kanehara, K., Akiyama, Y. & Ito, K. (2001). Characterization of the yaeL gene product and its S2P-protease motifs in *Escherichia coli*. *Gene* **281**, 71–79. doi:10.1016/S0378-1119(01)00823-X.
- Kanehara, K., Ito, K. & Akiyama, Y. (2002). YaeL (EcfE) activates the σ^E pathway of stress response through a site-2 cleavage of anti- σ^E , RseA. *Genes Dev* **16**, 2147–2155. doi:10.1101/gad.1002302.
- Kanehara, K., Ito, K. & Akiyama, Y. (2003). YaeL proteolysis of RseA is controlled by the PDZ domain of YaeL and a Gln-rich region of RseA. *EMBO J* **22**, 6389–6398. doi:10.1093/emboj/cdg602.
- Korndörfer, I. P., Dommel, M. K. & Skerra, A. (2004). Structure of the periplasmic chaperone Sklp suggests functional similarity with cytosolic chaperones despite differing architecture. *Nat Struct Mol Biol* **11**, 1015–1020. doi:10.1038/nsmb828.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176. doi:10.1016/0378-1119(95)00584-1.
- Kovacikova, G. & Skorupski, K. (2002). The alternative sigma factor σ^E plays an important role in intestinal survival and virulence in *Vibrio cholerae*. *Infect Immun* **70**, 5355–5362. doi:10.1128/IAI.70.10.5355-5362.2002.
- Lewis, C., Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Stevenson, A., Sherry, A., Kormanec, J. & Roberts, M. (2008). 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. *Microbiology* **154**, 979–988. doi:10.1099/mic.0.2007/011999-0.
- Lewis, C., Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Stevenson, A., Spencer, J., Farn, J., Kormanec, J. & Roberts, M. (2009). *Salmonella enterica* serovar Typhimurium HtrA: regulation of expression and role of the chaperone and protease activities during infection. *Microbiology* **155**, 873–881. doi:10.1099/mic.0.023754-0.
- Missiakas, D., Betton, J. M. & Raina, S. (1996). New components of protein folding in extracytoplasmic compartments of *Escherichia coli*

- SurA, FkpA and Skp/OmpH. *Mol Microbiol* **21**, 871–884. doi:10.1046/j.1365-2958.1996.561412.x.
- 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 σ^E in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* **226**, 307–314. doi:10.1016/S0378-1097(03)00600-1.
- Muller, C., Bang, I.-S., Velayudhan, J., Karlinsey, J., Papenfort, K., Vogel, J. & Fang, F. C. (2009). Acid stress activation of the σ^E stress response in *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **71**, 1228–1238. doi:10.1111/j.1365-2958.2009.06597.x.
- Nikolaus, T., Deiwick, J., Rappl, C., Freeman, J. A., Schröder, W., Miller, S. I. & Hensel, M. (2001). SseBCD proteins are secreted by the type III secretion system of *Salmonella* pathogenicity island 2 and function as a translocon. *J Bacteriol* **183**, 6036–6045. doi:10.1128/JB.183.20.6036-6045.2001.
- Osborne, S. E. & Coombes, B. K. (2009). RpoE fine tunes expression of a subset of SsrB-regulated virulence factors in *Salmonella enterica* serovar Typhimurium. *BMC Microbiol* **9**, 45. doi:10.1186/1471-2180-9-45.
- Pallen, M. J. & Wren, B. W. (1997). The HtrA family of serine proteases. *Mol Microbiol* **26**, 209–221. doi:10.1046/j.1365-2958.1997.5601928.x.
- 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. doi:10.1073/pnas.72.3.784.
- Redford, P. & Welch, R. A. (2006). Role of sigma E-regulated genes in *Escherichia coli* uropathogenesis. *Infect Immun* **74**, 4030–4038. doi:10.1128/IAI.01984-05.
- 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. doi:10.1016/S0167-7012(01)00237-8.
- Rhodium, V. A., Suh, W. C., Nonaka, G., West, J. & Gross, C. A. (2006). Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4**, e2. doi:10.1371/journal.pbio.0040002.
- Rizzitello, A. E., Harper, J. R. & Silhavy, T. J. (2001). Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. *J Bacteriol* **183**, 6794–6800. doi:10.1128/JB.183.23.6794-6800.2001.
- 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. doi:10.1128/IAI.73.1.459-463.2005.
- Rowley, G., Spector, M., Kormanec, J. & Roberts, M. (2006). Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol* **4**, 383–394. doi:10.1038/nrmicro1394.
- Schäfer, U., Beck, K. & Müller, M. (1999). Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins. *J Biol Chem* **274**, 24567–24574. doi:10.1074/jbc.274.35.24567.
- Schlapschy, M., Dommel, M. K., Hadian, K., Fogarasi, M., Korndörfer, I. P. & Skerra, A. (2004). The periplasmic *E. coli* chaperone Skp is a trimer in solution: biophysical and preliminary crystallographic characterization. *Biol Chem* **385**, 137–143. doi:10.1515/BC.2004.032.
- Shrestha, A., Shi, L., Tanase, S., Tsukamoto, M., Nishino, N., Tokita, K. & Yamamoto, T. (2004). Bacterial chaperone protein, Skp, induces leukocyte chemotaxis via C5a receptor. *Am J Pathol* **164**, 763–772.
- Sklar, J. G., Wu, T., Kahne, D. & Silhavy, T. J. (2007). Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev* **21**, 2473–2484. doi:10.1101/gad.1581007.
- 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. doi:10.1099/mic.0.28744-0.
- 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. doi:10.1128/IAI.68.3.1109-1115.2000.
- Thome, B. M. & Müller, M. (1991). Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for export. *Mol Microbiol* **5**, 2815–2821. doi:10.1111/j.1365-2958.1991.tb01990.x.
- Tokuda, H. (2009). Biogenesis of outer membranes in Gram-negative bacteria. *Biosci Biotechnol Biochem* **73**, 465–473. doi:10.1271/bbb.80778.
- Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A. & Sauer, R. T. (2003). OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**, 61–71. doi:10.1016/S0092-8674(03)00203-4.
- Walton, T. A. & Sousa, M. C. (2004). Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. *Mol Cell* **15**, 367–374. doi:10.1016/j.molcel.2004.07.023.
- Yasukawa, T., Kanei-Ishii, C., Maekawa, T., Fujimoto, J., Yamamoto, T. & Ishii, S. (1995). Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. *J Biol Chem* **270**, 25328–25331. doi:10.1074/jbc.270.43.25328.

Edited by: J. Cavet