The alternative sigma factor $\sigma^E$ ($rpoE$) is essential for survival in vivo of Salmonella Typhimurium but is dispensable during growth in the laboratory. We have been identifying $\sigma^E$-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 $\sigma^E$-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 $\sigma^E$ 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 $\beta$-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 $\sigma^E$-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 $H_2O_2$, 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.

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 $\sigma^E$ (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, $\sigma^E$ is essential for cell viability (De Las Peñas et al., 1997). In contrast, in S. Typhimurium...
and other pathogens, σE 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; Kovacikova & Skorupski, 2002). Maximal activation of the σE 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 σE 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 σE 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 σE from RseA allows transcriptional activation of σE-dependent genes, many of which have specific functions related to outer membrane or periplasmic homeostasis.

The σE 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 (YfO) 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 σE regulon members, including ours, Skp has been identified as σE-regulated (Dartigalongue et al., 2001b; Skovierova et al., 2006), whilst loss of Skp also results in induction of the σE 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 μg ampicillin ml⁻¹, 40 μg chloramphenicol ml⁻¹, 50 μg kanamycin ml⁻¹ or 50 μg 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 (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-rpoES4 or pAC7 (negative control), respectively, was grown in LB with chloramphenicol to early exponential phase (OD600 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 μg) were hybridized to 0.2 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 skpSTREV and the direct unlabelled primer skpSTD1R; probe 2 was an 892 bp DNA fragment prepared using the 5’ end-labelled reverse

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primer lpxDSTREV and the direct unlabelled primer skpSTDIR; probe 3 was a 1068 bp DNA fragment prepared using the 5' end-labelled reverse primer rseP-REV and the direct unlabelled primer lpxDSTREV and the direct unlabelled primer skpSTDIR; probe 4 was a 1242 bp DNA fragment prepared using the 5' end-labelled reverse primer yaeT REV and the direct unlabelled primer yaeT2; probe 5 was a 1554 bp DNA fragment prepared using the 5' end-labelled reverse primer rseP-2 and the direct unlabelled primer cdsA DIR. Oligonucleotides were 5'-labelled with 32P [ICN, 4500 Ci

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>SL1344</td>
<td>his, mouse-virulent</td>
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</tr>
<tr>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-5</td>
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</tr>
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<td>Rezuchova &amp; Kormanec (2001)</td>
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<td>pACYC-T7</td>
<td>CmR, pACYC184 plus T7 promoter from pAR2156</td>
<td>Yasukawa <em>et al.</em> (1995)</td>
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<td>pT-rpoE</td>
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<td>This study</td>
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Table 2. Oligonucleotides used in this study

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<td>cdsADR</td>
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<tr>
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<td>rpoENde</td>
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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 HindIII 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$^+$) 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 cdsDir and rseP2, which are external to the site of mutagenesis as well as internal primers within the Km$^+$ 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 Δskp::kan mutant (GVBl1367) 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$^+$ 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 htrAwx2ecoRI and htrAwx. The skp gene from S. Typhimurium SL1344 was amplified using primers skpw and skpvrNcoI. The resulting PCR products were purified, mixed in equal amounts and joined together using splicing by overlapping extension PCR with primers htrAwx2ecoRI and skpvrNcoI. The resulting fragment was then digested with EcoR1 and NcoI. Plasmid pBRI1MCS-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 μg gentamicin ml$^{-1}$. Clones were verified by DNA sequencing. The resulting plasmid was transformed into SL1344 Δskp (GVBl1367) to produce strain GVB 2225.

**Complementation of S. Typhimurium rseP-T.** To complement the rseP mutation, S. Typhimurium rseP-T was electroporated with plasmid pseP-I. This contains rseP under the control of the three S. Typhimurium rpoE promoters and a ribosome-binding site. pseP-I 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 Sall and NdeI at the beginning and the end of the promoter region. The PCR product was digested with Sall 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 OD$ob$. 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 10000 g. The pellet was washed twice with 50 mM Tris-buffered ethanol and recovered by centrifugation at 10000 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 μl. Female BALB/c mice (6–8 weeks old, Harlan) were used throughout. Initial assessment of Δskp virulence was carried out by using the well-described competitive index (CI) assay (Beuzon & 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 × 10$^8$ c.f.u.; skp mutant, ~5 × 10$^8$ 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. For oral infection, the inoculum [wild-type (WT), ~5 × 10$^8$ c.f.u.; skp mutant, ~5 × 10$^8$ 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 σ5 meant that we had to 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 σE-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
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 $\sigma^E$-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 $\sigma^E$-dependent yaeTp1 promoter was induced at stationary phase and by cold shock, conditions known to induce $\sigma^E$-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

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**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 $\mu$g 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.
yaeTp1 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 yaeTp1 promoter is dependent upon $\sigma^E$ in vivo. The sequence of the promoter (Fig. 2) is highly similar to the $\sigma^E$-consensus sequence, GGAACCTT-N15-GTCTAA (Skovierova et al., 2006).

In addition to the single $\sigma^E$-dependent yaeTp1 promoter, there are four other $\sigma^E$-independent promoters. Based on their sequences (Fig. 2), they would all appear to bind RNA polymerase containing the principal sigma factor $\sigma^70$, 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

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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/DDBJ 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 σE-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 SL1334 chromosome using λ Red mutagenesis (Datsenko & Wanner, 2000). We had no difficulty in replacing the skp gene with a KmR 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 σE from RseA for full activation of the σE pathway, which we know is not essential for S. Typhimurium viability (Humphreys et al., 1999).

From our transcriptional studies, the σE-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. Kormane, 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 inactive rseP (rseP-T) by deleting the first 426 bases of the rseP sequence, which retained the yaeTp1 and yaeTp2 promoters.

**RseP is required for σE activation and S. Typhimurium infection of mice**

rseP-T had no stress-induced σE activity as determined by S1 mapping of the σE-dependent rpoEP3 promoter. Loss of σE in Salmonella results, amongst other phenotypes, in loss of virulence in a murine typhoid model. This makes a good phenotypic check for deregulation of σE activity. The c.f.u. in the spleen of mice infected with the degS mutant is significantly different from that of the rpoEP3 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 σE 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 σE 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 conditions.
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 × 10^8 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 (Kornörfer 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 (Kornörfer 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

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**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 $\sigma^E$ itself, loss of functional Skp has also been shown to induce the $\sigma^E$ regulon (Missiakas et al., 1996).

In the current study we have shown that regulation of the rsePskypaeT 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 $\sigma^E$-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 $\sigma^E$-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 $\sigma^E$-dependent promoters in this locus (Dartigalongue et al., 2001b). However, none of these promoters fits the $\sigma^E$ 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 $\sigma^I$ 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 $\sigma^E$-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 *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 $\sigma^E$-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* ΔrpoE 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 $\sigma^I$, 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 $\sigma^E$ pathway independently of DegS (Muller et al., 2009). Those authors provide evidence that RseP and $\sigma^E$, 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 rsePyaeTska 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 rseP and skp, 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 $\sigma^I$ 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


Edited by: J. Cavet