Identification of the σ^E regulon of *Salmonella enterica* serovar Typhimurium

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The extracytoplasmic function sigma factor, σ^E , has been shown to play a critical role in virulence of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). The previously optimized two-plasmid system has been used to identify *S. Typhimurium* promoters recognized by RNA polymerase containing σ^E . This method allowed identification of 34 σ^E -dependent promoters that direct expression of 62 genes in *S. Typhimurium*, 23 of which (including several specific for *S. Typhimurium*) have not been identified previously to be dependent upon σ^E in *Escherichia coli*. The promoters were confirmed in *S. Typhimurium* and transcriptional start points of the promoters were determined by S1-nuclease mapping. All the promoters contained sequences highly similar to the consensus sequence of σ^E -dependent promoters. The identified genes belonging to the *S. Typhimurium* σ^E -regulon encode proteins involved in primary metabolism, DNA repair systems and outer-membrane biogenesis, and regulatory proteins, periplasmic proteases and folding factors, proposed lipoproteins, and inner- and outer-membrane proteins with unknown functions. Several of these σ^E -dependent genes have been shown to play a role in virulence of *S. Typhimurium*.

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INTRODUCTION

Serovars of *Salmonella enterica* are intracellular pathogens of vertebrates that cause a wide spectrum of diseases. *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) causes a typhoid-like systemic infection in mice and enteritis in humans and other animals. Within its host and in the environment, *Salmonella* species, like other bacteria, are exposed to a wide variety of stresses. To survive these detrimental conditions, bacteria have evolved a number of stress response systems, including the so-called extracytoplasmic stress response (ESR). In the related species *Escherichia coli*, the ESR has been shown to be governed by at least three partially overlapping signal transduction pathways: the CpxRA and BaeSR two-component systems and the extracytoplasmic function (ECF) sigma factor RpoE (σ^E) (Ruiz & Silhavy, 2005). The *rpoE* gene is located in an operon that includes three downstream genes, *rseA*, *rseB* and *rseC*. The *E. coli* *rpoE* gene is essential for cell viability and its expression is autoregulated and induced under conditions leading to the misfolding of periplasmic and outer-membrane proteins, such as heat-shock, and ethanol and osmotic stress.

The activity of σ^E is controlled by its specific membrane-bound anti-sigma factor, RseA, which, under non-stressed conditions, sequesters the majority of σ^E . In response to outer-membrane protein folding perturbations, RseA is cleaved by the successive action of two membrane proteases, DegS and YaeL (RseP), liberating the complex into the cytoplasm where RseA is degraded, freeing σ^E to complex with core RNA polymerase to govern expression of σ^E -dependent genes (reviewed by Alba & Gross, 2004). Two independent molecular genetic approaches have previously identified 58 members of the *E. coli* σ^E regulon, including periplasmic proteases and folding factors, several phospholipids and lipopolysaccharide (LPS) biosynthesis proteins, regulatory proteins, primary metabolism proteins and proteins with unknown function (Dartigalongue *et al.*, 2001; Rezuchova *et al.*, 2003). Recently, DNA microarray analysis after transient expression of *rpoE* in exponential- and early-stationary-phase *E. coli* has identified 156 genes that were significantly upregulated, including the previously reported 31 σ^E regulon genes (Kabir *et al.*, 2005). This approach increased the number of genes in the σ^E regulon to 183, although many of these may be indirectly dependent upon σ^E (Kabir *et al.*, 2005).

Unlike in *E. coli*, the *rpoE* gene in *S. Typhimurium* is not essential for cell viability, even at high temperature. However, *S. typhimurium* σ^E has been shown to be required for oxidative stress resistance, stationary-phase survival and

Abbreviations: ECF, extracytoplasmic function; ESR, extracytoplasmic stress response; σ^E , RNA polymerase holoenzyme containing σ^E ; OPG, osmoregulated periplasmic glucan(s); OPP, oligopeptide permease; TSP, transcription start point.

pathogenicity. *S. Typhimurium rpoE* mutants are defective in survival and proliferation in macrophage and epithelial cell lines, and are highly attenuated for virulence in a mouse model (Humphreys *et al.*, 1999; Kenyon *et al.*, 2002; Testerman *et al.*, 2002). The reduced virulence of the mutant is partially due to the increased sensitivity to reactive oxygen species produced by host macrophages (Humphreys *et al.*, 1999; Testerman *et al.*, 2002). *S. Typhimurium* σ^E also plays an important role in resistance to non-oxidative mammalian host defence mechanisms such as antimicrobial peptides (Humphreys *et al.*, 1999; Crouch *et al.*, 2005). Moreover, the *S. Typhimurium rpoE* gene has been shown to be up-regulated in *S. Typhimurium* within macrophages *in vitro* and murine tissues *in vivo* (Eriksson *et al.*, 2003; Rollenhagen *et al.*, 2004). These results indicate that the genes of the σ^E regulon should play roles in all these processes. Thus, identification and characterization of the *S. Typhimurium* σ^E regulon may reveal new genes involved in the virulence and survival of *S. Typhimurium* in the host.

Although organization of the *S. Typhimurium rpoE* operon resembles its counterpart in *E. coli*, its regulation is slightly different. Expression of *S. Typhimurium rpoE* is governed by three promoters, including one, *rpoEp3*, directly recognized by RNA polymerase holoenzyme containing σ^E ($E\sigma^E$). Like its *E. coli* counterpart, the *rpoEp3* promoter is partially induced by heat shock and osmotic stress, but it is most strongly induced by cold shock and entry into stationary phase (Miticka *et al.*, 2003). By using the σ^E -dependent *rpoEp3* promoter, we optimized the previously established *E. coli* two-plasmid system for the identification of promoters recognized by *S. Typhimurium* σ^E . The *S. Typhimurium rpoE* gene was cloned in an expression plasmid under the control of an inducible promoter and the *rpoEp3* promoter was cloned upstream of a reporter gene in a compatible promoter-probe plasmid. The promoter was active in the *E. coli* two-plasmid system only after induced expression of *S. Typhimurium rpoE*, with a transcription start point (TSP) identical to that in *S. Typhimurium* (Miticka *et al.*, 2003). In the present paper, we have used this optimized *E. coli* two-plasmid system to identify and locate *S. Typhimurium* σ^E -dependent promoters directing expression of genes which belong to the *S. Typhimurium* σ^E regulon. Moreover, the deduced functions of the identified genes are discussed in relation to the virulence of *S. Typhimurium*.

METHODS

Bacterial strains, plasmids and culture conditions. *S. Typhimurium* SL1344 (Hoiseth & Stocker, 1981) was used for chromosomal DNA preparation. *E. coli* XL-1 Blue (Stratagene) was used as a host for cloning experiments. The *E. coli* plasmid pSB40 (Park *et al.*, 1989) was kindly provided by Dr M. K. Winson, University of Nottingham. The expression plasmid pAC7 has been described by Rezuchova & Kormanec (2001). Plasmid pAC-rpoEST4 containing the *S. Typhimurium rpoE* gene under the control of the arabinose-inducible P_{BAD} promoter has been described by Miticka *et al.* (2003). For RNA isolation, *E. coli* with the corresponding plasmids was inoculated in LB medium (Ausubel *et al.*, 1995) supplemented

with ampicillin (50 $\mu\text{g ml}^{-1}$) and chloramphenicol (40 $\mu\text{g ml}^{-1}$) and grown at 37 °C to exponential phase ($OD_{600}=0.3$). Expression of *S. Typhimurium rpoE* was induced for 3 h with 0.0002% (w/v) arabinose. To grow *S. Typhimurium* with *rpoE* artificially expressed for RNA isolation, *S. Typhimurium* SL1344 containing pAC-rpoEST4 or pAC7 (as negative control) were grown in LB with 40 μg chloramphenicol ml^{-1} to exponential phase ($OD_{600}=0.24$) and expression of *rpoE* was induced for 3 h with 0.2% (w/v) arabinose. Conditions for *E. coli* growth and transformation were as described by Ausubel *et al.* (1995).

DNA manipulations. DNA manipulations in *E. coli* were performed as described by Ausubel *et al.* (1995). Nucleotide sequencing was performed by the chemical method (Maxam & Gilbert, 1980) and by the dideoxy chain-termination method (Sanger *et al.*, 1977), using a TaqTrack kit (Promega). An *S. Typhimurium* SL1344 genomic library was prepared by cloning 0.5–1.2 kb partial *Sau3AI* chromosomal DNA fragments into the *Bam*HI site of pSB40. About 120 000 original clones obtained from the transformation of *E. coli* XL-1 Blue were used for total plasmid isolation by using a Qiagen plasmid purification kit. The clones were statistically checked for the presence of insert and all the picked clones contained fragments in the range 0.5–1.2 kb.

Detection of *E. coli* clones containing the *rpoE*-dependent promoter fragment. The *S. Typhimurium* SL1344 genomic library was transformed into *E. coli* XL-1 Blue containing the compatible plasmid pAC-rpoEST4. The clones were selected on LBACX plates (LB medium with 5 g lactose l^{-1} , 100 μg ampicillin ml^{-1} , 40 μg chloramphenicol ml^{-1} , 20 μg X-Gal ml^{-1}) with 2 μg arabinose ml^{-1} as described by Miticka *et al.* (2003). The colonies were screened after 24 h growth at 37 °C. Blue clones were inoculated in parallel onto two LBACX plates containing either 2 μg arabinose ml^{-1} (LBACX-ARA) or 2 mg glucose ml^{-1} (LBACX-GLU), respectively. Clones that were blue on LBACX-ARA and white on LBACX-GLU were inoculated into 1 ml LB with 100 μg ampicillin ml^{-1} and grown overnight at 37 °C. Cells were pelleted, resuspended in 200 μl STE buffer (0.1 M NaCl, 10 mM Tris/HCl, pH 8, 1 mM EDTA) with 0.5 mg lysozyme ml^{-1} , incubated for 5 min at room temperature, boiled for 1.5 min and centrifuged for 10 min at 16 000 g. One microlitre of supernatant was transformed in parallel into *E. coli* XL-1 Blue strains harbouring either pAC-rpoEST4 or pAC7 and plated onto LBACX-ARA.

Isolation of RNA and S1-nuclease mapping. After finishing growth, cell suspensions of *E. coli* or *S. Typhimurium* were immediately poured into 50 ml Falcon tubes containing about 15 ml crushed ice prechilled to -80°C , then the cells were centrifuged, washed with DEPC-treated ice-cold 0.15 M NaCl and total RNA was prepared as described by Kormanec (2001). High-resolution S1-nuclease mapping was performed according to Kormanec (2001). Samples (40 μg) of RNA were hybridized to approximately 0.02 pmol of a suitable DNA probe labelled at one 5' end with [γ - ^{32}P]ATP [approx. 3×10^6 c.p.m. (pmol probe) $^{-1}$] and treated with 120 U S1-nuclease. The probes for S1-nuclease mapping of the proposed *S. Typhimurium* σ^E -dependent promoters in the *E. coli* two-plasmid system were prepared by PCR amplification from the corresponding pEST plasmid (pEST1–pEST124) isolated from the positive clone using the 5' end-labelled universal oligonucleotide primer –47 from the *lacZx*-coding region of the pEST plasmid, and primer mut80 from the 5' region flanking the *Bam*HI cloning site of pSB40 (Table 1). The probes for S1-nuclease mapping for *in vivo* verification in *S. Typhimurium* were prepared by PCR amplification from the corresponding pEST plasmid using the 5' end-labelled internal reverse primer from the corresponding coding region (Table 1), and the direct primer mut80. Oligonucleotides were labelled at their 5' ends with [γ - ^{32}P] (4500 Ci mmol $^{-1}$; ICN) and T4 polynucleotide kinase. The labelled DNA fragments were isolated from

Table 1. Primers used in this study

Name	Sequence (5'→3')	Characteristic
–47 mut80	CGCCAGGGTTTCCCAGTCACGAC GGGTTCCGCGCACATTTCCCCG	Reverse primer from <i>lacZx</i> of pSB40 Direct primer from 5' region flanking the <i>Bam</i> HI site of pSB40
YggTRev	CAGAACATTTAGCGCCGGCAG	Reverse primer from the <i>yggT</i> gene
YraPRev	GCGAAAATGCCTTCATGTGTAC	Reverse primer from the <i>yraP</i> gene
YgiMRev	CTTCAGCATGAGAGACGGCG	Reverse primer from the <i>ygiM</i> gene
TolRRev	CAATGCTTCGGCAATACCCG	Reverse primer from the <i>tolR</i> gene
RpoDRev	GGGCATACTTATATTTTGGC	Reverse primer from the <i>rpoD</i> gene
S1251Rev	CTATCAATACGGTTGAAACG	Reverse primer from the <i>stm1251</i> gene
S1250Rev	CCTTTTGGGAGGAAATATGGTCCG	Reverse primer from the <i>stm1250</i> gene
PsdRev	GTTTCGGCAGAATGTATTGTAGCG	Reverse primer from the <i>psd</i> gene
YcbKRev	GGGTCGAGAGTGTGGCAAACG	Reverse primer from the <i>ycbK</i> gene
FkpARev	CGTGCATAGCAACGGCCATCG	Reverse primer from the <i>fkpA</i> gene
PrtRev	GCGGTTGCCAACCGATATCC	Reverse primer from the <i>ptr</i> gene
SurARev	CCCGACGGTATTCGAGGCTGC	Reverse primer from the <i>surA</i> gene
YabIRev	CCGTGCCGGGTAAAATCAACC	Reverse primer from the <i>yabI</i> gene
YfiORev	GCCAGAAACAGGCTCAACG	Reverse primer from the <i>yfiO</i> gene
YiiDRev	CCAGCGTAACTGCGACACC	Reverse primer from the <i>yiiD</i> gene
DedDRev	CCGTCCAGCAGACCGGGAAGC	Reverse primer from the <i>dedD</i> gene
YfeKRev	CCTTTTCTGCGCCAGCGC	Reverse primer from the <i>yfeK</i> gene
SbmARev	ACGGCGATCAGCGCCCAACAAACGCCG	Reverse primer from the <i>sbmA</i> gene
RpoHRev	CGCCGCCCGGATATAAGATTCC	Reverse primer from the <i>rpoH</i> gene
HtrARev	GCCAAACCTAAACTCAGAGCCAG	Reverse primer from the <i>htrA</i> gene
LpxPRev	CCAGTAGCGCGGGTGCAAAAACG	Reverse primer from the <i>lpxP</i> gene
YaeTRev	CCCAGTGACGTAAATCAAGCG	Reverse primer from the <i>yaeT</i> gene
OppARev	GCTTGTCGGCTAACTGAACG	Reverse primer from the <i>oppA</i> gene
YggNRev	CCGTTTTACCTTTACCTGC	Reverse primer from the <i>yggN</i> gene
YfeYRev	CCTTGCTCAGTGACTTCTGTGC	Reverse primer from the <i>yfeY</i> gene
YeaYRev	GGATAGTCACGCAACCGCTC	Reverse primer from the <i>yeaY</i> gene
YiaDRev	CGGTGTAAGGGTTTGTGTGC	Reverse primer from the <i>yiaD</i> gene
FusARev	ECATCCAGTCCATGGTAGCTGC	Reverse primer from the <i>fusA</i> gene
EnoRev	GATCACCGACTTATAGGCATCC	Reverse primer from the <i>eno</i> gene
PlsBRev	GAATAGACTTGCTTTTACC	Reverse primer from the <i>plsB</i> gene
YdcGRev	CTGAATGCTGTTATAGGCCTGC	Reverse primer from the <i>ycdG</i> gene

polyacrylamide gels as described by Kormanec (2001). 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 (Maxam & Gilbert, 1980).

RESULTS AND DISCUSSION

Identification of the *S. Typhimurium* promoters recognized by $E\sigma^E$ using the *E. coli* two-plasmid system

To identify *S. Typhimurium* σ^E -dependent promoters, we used the optimized *E. coli* two-plasmid screening system that was successfully used for the identification of the *E. coli* σ^E regulon (Rezuchova *et al.*, 2003). This method assumes that the *E. coli* RNA polymerase core enzyme will interact with a particular heterologous sigma factor expressed from one plasmid, and that the resulting holoenzyme can

recognize a promoter present in a library of chromosomal fragments cloned in the second compatible plasmid, upstream of a reporter gene. This *E. coli* two-plasmid system was optimized using the *S. Typhimurium* σ^E -dependent *rpoEp3* promoter. The *S. Typhimurium* *rpoE* gene was cloned into expression plasmid pAC7 under the control of an arabinose-inducible P_{BAD} promoter, resulting in plasmid pAC-rpoEST4. Following arabinose-induced expression of *S. Typhimurium* *rpoE*, *E. coli* RNA polymerase holoenzyme containing *S. Typhimurium* σ^E ($E\sigma^E$) was able to recognize the *rpoEp3* promoter cloned upstream of the *lacZx* reporter gene in the second compatible plasmid. Moreover, the transcription of the *rpoEp3* promoter was initiated from the identical TSP as in *S. Typhimurium* (Miticka *et al.*, 2003). These results indicated that this optimized *E. coli* two-plasmid system could be used for identification of *S. Typhimurium* σ^E -dependent promoters. For this purpose, an *S. Typhimurium* genomic library cloned into

pSB40 was used to transform *E. coli* XL-1 Blue containing pAC-rpoEST4. After screening of about 120 000 colonies on LBACX-ARA plates, 5040 blue clones that represented promoters active in *E. coli* (including σ^E -dependent promoters) were picked up. After further selection of the identified blue clones on LBACX-ARA and LBACX-GLU plates, the plasmids were isolated from 1020 clones and transformed in parallel into *E. coli* XL-1 Blue with pAC7 and pAC-rpoEST4, respectively. Colonies were screened on LBACX-ARA plates. Clones containing plasmids with σ^E -dependent promoters were blue in *E. coli* XL-1 Blue with pAC7-rpoEST4 and white in *E. coli* XL-1 Blue containing pAC7. Clones with σ^E -independent promoters were blue in both strains. With this last screen we identified 124 positive clones containing putative σ^E -dependent promoters (plasmids pEST1–pEST124). Sequencing of the DNA fragments revealed 34 representatives. Although the quality of the library was high (about 120 000 original clones correspond to a calculated probability greater than 0.99999), we cannot rule out that the *S. Typhimurium* library used covered the complete genome. Several representatives of the σ^E -dependent promoters were found more than 10 times and some were found only once. Therefore, the number of identified σ^E -dependent promoters may not be complete.

Characterization of *S. Typhimurium* promoters recognized by $E\sigma^E$

To locate the TSP of the identified *S. Typhimurium* σ^E -dependent promoters, high-resolution S1-nuclease mapping was performed using RNA isolated from *E. coli* XL-1 Blue, containing a corresponding pEST plasmid bearing a particular σ^E -dependent promoter and pAC-rpoEST4, grown to exponential phase and induced by arabinose. The 5'-labelled DNA probes were prepared from the corresponding pEST plasmid with external primers, enabling the location of the putative σ^E -dependent promoter only in the pEST plasmid-bearing DNA fragment. In all cases, RNA-protected fragments were identified only using RNA from *E. coli* XL-1 Blue with the corresponding pEST plasmid and pAC-rpoEST4 grown under conditions inducing *S. Typhimurium* *rpoE*. No RNA-protected fragment was identified with a control RNA from *E. coli* containing a particular pEST plasmid and pAC7 grown under similar conditions. To investigate the activities of these putative σ^E -dependent promoters in their chromosomal location in *S. Typhimurium*, and to confirm their dependence upon σ^E , high-resolution S1-nuclease mapping was performed using the 5'-labelled probes prepared from the corresponding pEST plasmids using internal reverse primers from the coding regions of the corresponding σ^E -dependent *S. Typhimurium* genes and RNA isolated from *S. Typhimurium* SL1344 containing pAC-rpoEST4 or pAC7, respectively, grown to exponential phase and induced for 3 h with arabinose. RNA-protected fragments were identified using RNA isolated from *S. Typhimurium* SL1344 containing pAC-rpoEST4, and grown to exponential phase with *rpoE* expression artificially induced with arabinose

(Fig. 1, lanes 1). No RNA-protected fragment was identified with control RNA from *S. Typhimurium* SL1344 containing pAC7, grown to exponential phase and also induced with arabinose (Fig. 1, lanes 2). The TSPs of the identified promoters were in the identical positions as for the σ^E -dependent promoter in the *E. coli* two-plasmid system located on the corresponding pEST plasmid. Thus, these results indicated that the chromosomally located promoters are dependent *in vivo* on σ^E in *S. Typhimurium*. By using this strategy, 34 σ^E -dependent promoters were localized and verified *in vivo* in *S. Typhimurium*. Comparison of the nucleotide sequences upstream of the identified TSPs (Fig. 2) revealed a consensus promoter sequence that is similar to that of σ^E of *E. coli* (Rezuchova *et al.*, 2003). Interestingly, based on the generated sequence logo (Fig. 2b), another residue, a G preceding the -10 region, appeared to be conserved in the σ^E -dependent promoters, thus suggesting a new σ^E -consensus sequence, GGAAGTT-N₁₅-GTCTAA. The generated logo and the conservation of nucleotides within the -35 and -10 regions (Fig. 2) correlates well with our experimental analysis of the importance of specific bases within the *S. Typhimurium* σ^E -dependent *rpoEp3* promoter for binding with $E\sigma^E$. This mutagenesis analysis identified the bases shown in upper case letters as the most important in the -35 (ggAACTT) and -10 (TctaA) regions (Miticka *et al.*, 2004). Interestingly, as in *E. coli* (Rezuchova *et al.*, 2003), in almost all cases (except *surAp*), strictly conserved spacing between the -10 and -35 recognition sites was found in *S. Typhimurium* σ^E -dependent promoters (Fig. 2). In several cases, additional σ^E -independent promoters were identified, in addition to the corresponding σ^E -dependent promoter, that direct expression of the corresponding gene of the *S. Typhimurium* σ^E regulon (Fig. 1).

Identification of *S. Typhimurium* σ^E -dependent genes

Comparison of the nucleotide sequence downstream of identified promoters with the complete sequence of *S. Typhimurium* LT2 (<http://genomeold.wustl.edu/projects/bacterial/styphimurium/>) and almost completed sequence of *S. Typhimurium* SL1344 (www.sanger.ac.uk/Projects/Salmonella) revealed the genes directed by the identified *S. Typhimurium* σ^E -dependent promoters (Table 2). The 34 identified σ^E -dependent promoters control expression of 62 genes found in both *Salmonella* genomes, including 18 single genes and 13 proposed operons. Possible operon structures of the σ^E -dependent genes were predicted on the basis of close gene arrangements and transcription direction in the genomic sequence of *S. Typhimurium* (in many cases ORFs in operons were translationally coupled). One operon (*stm1250*, *stm1251*) was controlled by two tandem σ^E -dependent promoters. Interestingly, 13 σ^E -dependent promoters were located in the coding region of the upstream convergent genes. Based on these data, these 62

σ^E -dependent genes probably constitute the σ^E regulon in *S. Typhimurium*.

Members of the σ^E regulon common in *S. Typhimurium* and *E. coli*

Three independent approaches have already identified 183 σ^E -dependent genes in *E. coli* (Dartigalongue *et al.*, 2001; Kabir *et al.*, 2005; Rezuchova *et al.*, 2003). As in *E. coli*, the genes regulated by *S. Typhimurium* σ^E fall into similar functional categories, including periplasmic proteases and folding factors, proteins involved in cell membrane integrity and in phospholipid and LPS biosynthesis, regulatory proteins, primary metabolism proteins and membrane or periplasmic proteins of unknown function (Table 2). Of the 62 identified *S. Typhimurium* σ^E -dependent genes, 39 orthologues (*rpoE*, *rseA*, *rseB*, *rseC*, *rpoH*, *rpoD*, *fusA*, *tufA*, *htrA*, *recB*, *surA*, *pdxA*, *ksgA*, *apaG*, *apaH*, *fkpA*, *plsB*, *psd*, *yjeP*, *lpxP*, *yaeT*, *hlpA*, *lpxD*, *yfiO*, *tolA*, *tolB*, *pal*, *ybgF*, *yabI*, *ycbK*, *ycbL*, *yeaY*, *yfeY*, *yiiD*, *sbmA*, *yaiW*, *yraP*, *ygiM*, *yggN*) have been shown previously to be σ^E -dependent in *E. coli*. Recently, two of the identified members of the σ^E regulon, YaeT and YfiO, have been shown to form a multicomponent complex together with YfgL and NlpB proteins which is essential for the assembly of proteins in the outer membrane of *E. coli* (Wu *et al.*, 2005), thus assigning a role of these two previously uncharacterized proteins in outer-membrane biogenesis.

Three of these σ^E -dependent genes have hitherto been shown to be involved in *Salmonella* virulence. The *surA* gene, encoding a periplasmic peptidylprolyl-*cis-trans*-isomerase (PPIase) involved in protein folding, has a role in adherence and invasion of host eukaryotic cells. Furthermore, the *S. Typhimurium* *surA* mutant was attenuated when administrated orally or intravenously to BALB/c mice and the *S. Typhimurium* *surA* mutant demonstrated potential as a vaccine candidate (Sydenham *et al.*, 2000). In contrast, the other σ^E -dependent PPIase-encoding gene, *fkpA*, has only a minor effect on the ability of *S. Typhimurium* to invade and survive within epithelial cells and macrophages and cause infection in mice. However, the effect of the *fkpA* mutation on *S. Typhimurium* virulence was more profound if the mutation was combined with a mutation in *surA*, or in another member of the σ^E regulon, *htrA* (Humphreys *et al.*, 2003). The *htrA* (*degP*) gene encodes a periplasmic protease essential for degradation of damaged proteins. In *E. coli*, HtrA is required for survival at high temperatures (Strauch *et al.*, 1989); in contrast, *S. typhimurium* *htrA* mutant strains are not temperature-sensitive, but are more sensitive to oxidizing agents and are required for survival within macrophages and for virulence in mice (Johnson *et al.*, 1991; Humphreys *et al.*, 1999). However, the difference in *S. Typhimurium* *rpoE* and *htrA* mutants in terms of the degree of their attenuation in mice and their sensitivity to noxious agents indicated that additional genes in the σ^E regulon should play a critical role in virulence and in combating a variety of stresses (Humphreys *et al.*, 1999).

Differentially regulated genes of the σ^E regulon in *S. Typhimurium* and *E. coli*

Intriguingly, the σ^E -dependent promoters of several *S. Typhimurium* genes were different to their counterparts previously identified in *E. coli*. The expression of the *rpoE*, *rseA*, *rseB*, *rseC* operon is governed by a σ^E -dependent promoter located in an almost identical position to a highly similar sequence (identical –35 and –10 conserved regions) in both *S. Typhimurium* and *E. coli* (Miticka *et al.*, 2003). Likewise, *S. Typhimurium* σ^E -dependent promoters *rpoHp*, *htrAp*, *sbmAp*, *fkpAp*, *fusAp*, *psdp*, *lpxP*, *yeaYp* and *yggNp* were highly similar (with almost identical –35 and –10 conserved regions) and located in almost identical positions to their counterparts in *E. coli*. However, the sequences and locations of both *S. Typhimurium* σ^E -dependent *rpoDp* promoters were different from their counterpart (*rpoDp3*) in *E. coli* (Dartigalongue *et al.*, 2001). We found that the reported *E. coli* σ^E -dependent *rpoDp3* promoter is located in a similar position to the previously located σ^H -dependent promoter in the *E. coli* *rpoD* gene (Taylor *et al.*, 1984). Moreover, we have identified this σ^H -dependent promoter in an identical position in *S. Typhimurium* (Fig. 1). A similar discrepancy has been found for the *S. Typhimurium* σ^E -dependent promoters *yfiOp*, *yraPp* and *ygiMp*. The σ^E -dependent promoters of all their *E. coli* counterparts (*ecfDp*, *ecfHp* and *ecfGp*) have been located further downstream (Dartigalongue *et al.*, 2001) and display only very weak similarity to the σ^E consensus sequence (Rezuchova *et al.*, 2003; Miticka *et al.*, 2004). The signals located by Dartigalongue *et al.* (2001) may thus correspond to the premature termination of the reverse transcriptase, as they used primer extension analysis for the location of the σ^E -dependent promoters. In our case, we verified σ^E -dependent promoters using the more reliable S1-nuclease mapping technique (Kormanec, 2001). Moreover, we found sequences highly similar to *S. Typhimurium* σ^E -dependent promoters (with identical –35 and –10 regions) in similar positions upstream of *E. coli* genes, suggesting they may correspond to the σ^E -dependent promoters in this species. However, we cannot rule out the possibility that these σ^E -dependent genes are differentially expressed in the two species. This also may be the case for *surA* and the *yaeL* (*ecfE*), *yaeT* (*ecfK*), *hlpA* (*skp*), *lpxD* operon. In *E. coli*, expression of *surA* is proposed to be governed by a σ^E -dependent promoter (which does not fit the σ^E consensus sequence) 176 bp upstream of the *imp* (*ostA*) gene that is located upstream of *surA* (Dartigalongue *et al.*, 2001). However in *S. Typhimurium*, the σ^E -dependent *surAp* promoter has been located at the 3' end of the *imp* (*ostA*) coding region.

In the case of the σ^E -dependent *yaeL* (*ecfE*), *yaeT* (*ecfK*), *hlpA* (*skp*), *lpxD* operon in *E. coli*, three proposed σ^E -dependent promoters were identified, none of which fit the σ^E consensus sequence (Dartigalongue *et al.*, 2001). The first proposed σ^E -dependent promoter, *ecfEp*, was located upstream of *yaeL* (*ecfE*), the second, *skpp*, was located upstream of the *hlpA* (*skp*) gene, and the third, *lpxDp2*, was located at

the end of the *hlpA* (*skp*) coding region, upstream of the *lpxD* gene (Dartigalongue *et al.*, 2001). However, in *S. Typhimurium*, we have identified only one σ^E -dependent promoter in this region, *yaeTp*, which is located in the *yaeL* coding region upstream of the *yaeT* gene. We have analysed the whole of the *S. Typhimurium yaeL, yaeT, hlpA, lpxD* operon region for other potential σ^E -dependent promoters but we were unable to identify any σ^E -dependent promoters in the regions corresponding to the proposed *E. coli* σ^E -dependent promoters, although there were several σ^E -independent promoters (data not shown). As with the previous cases, we have identified sequences highly similar to the σ^E -dependent promoter *yaeTp* (with almost identical conserved -35 and -10 regions) in a similar position in *E. coli*, indicating that this is likely to be the σ^E -dependent promoter directing expression of *yaeT* and downstream genes in *E. coli*. However, as for the previous promoters, we cannot rule out that there may be differences in the regulation of σ^E -dependent genes between *S. Typhimurium* and *E. coli*.

Members of the σ^E regulon specific for *S. Typhimurium*

We identified 23 *S. Typhimurium* σ^E -dependent genes (*ptr*, *recD*, *tolR*, *oppA*, *oppB*, *oppC*, *oppD*, *oppF*, *stm1741*, *eno*, *yggT*, *yggU*, *yggV*, *yggW*, *yjfO*, *yjfN*, *yiaD*, *dedD*, *ydcG*, *yfeK*, *yfeL*, *stm1250*, *stm1251*) that have not been previously identified to be dependent upon σ^E in *E. coli*. The inferred functions of some of these new members of σ^E regulon fall broadly into the same categories as previously described for the σ^E regulon (Dartigalongue *et al.*, 2001; Kabir *et al.*, 2005; Rezuchova *et al.*, 2003). Interestingly, in addition to the well characterized periplasmic serine protease HtrA (DegP), another periplasmic protease, Protease III, belongs to the σ^E regulon in *S. Typhimurium*. Protease III (Pitriylsis), the product of the *ptr* gene, is a periplasmic metalloprotease with specificity towards insulin and other low-molecular-mass substrates. The physiological role of Protease III is not known (Dykstra & Kushner, 1985; Swamy & Goldberg, 1982). It is thought that Protease III is involved in the turnover of proteins in the periplasmic space (Baneyx & Georgiou, 1991; Betton *et al.*, 1998; Cornista *et al.*, 2004). Thus, increased levels of Protease III may be needed after envelope stress. Expression of the *ptr* gene has been partially characterized in *E. coli*. A single promoter, 127 bp upstream from the start codon of *ptr*, has been identified in the

upstream region (Claverie-Martin *et al.*, 1987). Interestingly, no signal corresponding to this promoter region was identified in *S. Typhimurium*, although another σ^E -independent promoter was identified downstream of the σ^E -dependent *ptrp* promoter in the *recC* coding region 582 bp upstream from the start codon of *ptr* (data not shown). This indicates that *ptr* is differentially expressed in *E. coli* and *S. Typhimurium* and that *ptr* may not belong to the σ^E regulon in *E. coli*. As in *E. coli*, the *S. Typhimurium ptr* gene is intriguingly located between the *recC* and *recBD* genes which encode subunits of exonuclease V involved in DNA repair and genetic recombination. As the stop and start codons of *ptr*, *recB* and *recD* overlap, it is suggested that these genes may be part of an operon. The σ^E -dependent *ptrp* promoter may therefore also regulate expression of the downstream *recBD* genes, indicating a new role for the σ^E regulon in DNA repair and recombination. Actually, the *recB* gene has been recently found to be dependent upon σ^E in *E. coli* (Kabir *et al.*, 2005). Interestingly, mutants of *S. Typhimurium* lacking the *recBC* function are avirulent in mice and unable to grow inside macrophages, and it has been suggested that *S. Typhimurium* uses this RecBCD recombination pathway to repair DNA double-strand breaks produced during growth inside macrophages (Buchmeier *et al.*, 1993). Thus, *recBC* may be additional genes in the σ^E regulon that have a critical role in virulence.

One of the identified *S. Typhimurium* σ^E -dependent promoters has been located in the coding region of the *tolQ* gene in the *ybgC, tolQ, tolR, tolA, tolB, pal, ybgF* gene cluster. In *E. coli*, the genes in this cluster appear to be transcribed from two constitutive promoters, one immediately upstream of *ybgC* and the other upstream of *tolB*, and producing two transcripts: *ybgC, tolQRAB, pal, ybgF* and *tolB, pal, ybgF* (Vianney *et al.*, 1996). The *tolQRAB* and *pal* genes are conserved in most Gram-negative bacteria and encode proteins of the Tol-Pal system that are implicated in the maintenance of cell envelope integrity and in the transport of newly synthesized components through the periplasm. This system has also been found to facilitate the uptake of filamentous phage DNA and group A colicins. No obvious phenotypes have been assigned to *ybgC* and *ybgF*, which encode cytoplasmic and periplasmic proteins, respectively (Lazzaroni *et al.*, 1999; Cascales & Lloubes, 2004). Recently, it has been shown that the TolA protein is required for the correct surface expression of the *E. coli* O7 antigen, thus demonstrating a role of the Tol-Pal system in LPS

Fig. 1. Examples of TSP determination for *S. Typhimurium* σ^E -dependent promoters by high-resolution S1-nuclease mapping. The particular 5'-labelled DNA fragment was hybridized with 40 μ g RNA isolated from exponentially grown *S. Typhimurium* SL1344 containing pAC-rpoEST4 (lanes 1) or pAC7 (lanes 2) and induced for 3 h with 0.2% arabinose. The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G+A (lane A) and T+C (lane T) sequencing ladders derived from end-labelled fragments (Maxam & Gilbert, 1980). Thin horizontal arrows indicate the positions of RNA-protected fragments and thick angled arrows indicate the nucleotide corresponding to TSP. Before assigning the TSP, 1.5 nt was subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from S1-nuclease digestion and the chemical sequencing reactions. In some cases, the thick angled arrows indicate σ^E -independent promoters. All S1-nuclease mapping experiments were performed twice with independent sets of RNA with similar results.

(a)

		-35		-10	TSP
<i>rpoEp3</i>	ACAAACAAAAACAAATGCGTAAC	GGA ACTT	TACGAAACATAGACA	CTCT AA	CCTGTT G
<i>rpoHp</i>	GCAAATTATGCACAGTGTACAT	TGA ACTT	GTGGATAAAATCACT	GTCT GA	TAAAA
<i>rpoDp1</i>	TATGAGGCTCAGCAATATAGTGC	TGA ACCG	CAACGCTTGCTTGTG	GTC GAA	GGTTAT A
<i>rpoDp2</i>	TTGGCTTATTTAAAGAATTGGTC	AAA ACTT	GTTTGGCTCAGCCAG	GTCT TA	CTACC
<i>fusAp</i>	TCCATGGCTCTGCGCCTGGCGAA	CGA ACTT	TCTGATGCTGCAGAC	AA CAAA	GGT A
<i>enop</i>	TTATATTTGTAAACGATTACAGCT	TGA ACTG	TCCGGAAGCTAACCT	GTCT GA	ATGGG A
<i>ptrp</i>	TTAATCAGCGTGGCGCAAGGTGT	GCA ACTT	TGGCTGGAACACCTT	GTCT AT	TGTGCC A
<i>htrAp</i>	ACTTTTTCCAGAACTTTATTCC	GGA ACTT	CGCGTTATAAAATGA	ATCT GA	CGTAC A
<i>fkpAp</i>	ACAGCAACCATTACGCGCACTAC	GAA ACTA	ATTTAAACAAAAAGA	GTCT GA	AAAG A
<i>surAp</i>	CGCATTATGATGATGCCGCCGT	TGA ACGT	TTTAACGTTTCTGTTG	GTCA AA	TCTACTAT T
<i>plsBp</i>	CAGTGGTATTATTGGCCATCAAA	AGA ACCT	TTTTACATTATTAAC	GTCA AT	GACAT T
<i>psdP</i>	TTCGGCTTATGGCATCTGGAGCC	GGA ACAA	ATCACCAGGGCTTT	GTC GAA	TTT C A
<i>lpxPp</i>	GACTTAGTTTTACACTCCTTACG	GGA ACCA	TTGTCGTACATGATG	GCCT AA	CTAT A
<i>yaeTp</i>	CAGGCGCAAATTGCGCCTGGTAC	GGA ACTT	AAAGCCGTTGATGGT	ATC GAA	ACCCCT G
<i>yfiOp</i>	TATACGCGAACGTGAATAATCCG	GGA ACTT	TTCGGCCAAAGCCTG	ATCT AA	CGCT T
<i>dedDp</i>	GGCGCGACGGCGGAACAACCTCT	GGA ACAT	CTGGGGAAAAGGGAAT	GTCT AT	GAT G
<i>oppAp</i>	TTGTAGAAGATTTACATTGTTG	ATA AGTA	TTTTCATTTAGATGG	TTGT CA	GAAAT A
<i>sbmAp</i>	AACAATTCTGTTGAGCAGAAATG	CGA ACTA	AGCGTCACAACGTAG	GTC ACA	ATGCC TGGT
<i>yeaYp</i>	TTGCGTAACGAAGTGGCGTGGAA	GAA ACTT	CCCGGCAAAGAATGA	CTCT CA	CTACT A
<i>stm1250p</i>	CGTTTCATACGGTATCTGTCAGT	GAA ACTA	TTTCTTCAAATGATG	GTCT TTT	TTAT T
<i>stm1251p</i>	AGTTTTTTGTTTCAGCGGIGACT	GAA ACAT	ATTTTGTTCCTGGTG	GTCT TA	CCGCC A G
<i>yfeYp</i>	TTTTTCCCTTGAGAAAGAATAAC	GGC ACTT	TTTGGCGAACCCGCA	CTCC AA	CCA A
<i>tolRp</i>	AGACGCGATAGTCTGTCGGGCTC	GGA ACAA	ATCTTTTATAGCGGG	TTCA AA	GAGTT TG
<i>yabIp</i>	CAGGGCCGGATGTGAATAAAAAG	TGA ATGA	TGTGTCCACAAAGAT	GTC ATA	AGCCG T
<i>ycbKp</i>	TGAACAATTAATCAGGTAAATGA	AGA AGTT	CTGGTAAATATGTTG	TCCT AA	TGTT T
<i>ydcGp</i>	GGCGACCAACAATTGGATTATTA	ATG ATTT	TGTCTAGAGTGAGCG	GTC AGA	AAC G
<i>yfeKp</i>	ACAGAAATAACCACACCAGGAGT	GCA ACTT	ATTACCCGGCTAACG	GTCT GA	GGTG C
<i>yggNp</i>	AACCTTGAAGAAGTCCGAACCAG	CGA ACTT	TTCGACGTTTGGTGG	GA CTAA	TAAG T
<i>yggTp</i>	AGCGAAAGTGAAGGCCTGCACT	GGC ACTT	TATTGGCCCTTTACA	GTCC AA	CAAA A
<i>ygiMp</i>	GTCACATCTGGGAAGATTCTTGG	CGA ACTT	ACCCGCATTATTTTTT	GTC AGT	AGATAG T
<i>yiaDp</i>	TTACAAATTTTCAAGTTTTCCCT	GGA ATTT	TCTTCATGTGCAGAC	GTC ATA	ATCA ACC
<i>yiiDp</i>	GAGGTGACGGGTAAAATCGGCCC	AGG ACTT	TTGGTGTATTTGGGT	GTC GAA	AAAG A
<i>yjfOp</i>	ATCGCGTGACAGGTAACACTTCG	GGA ACTT	TCCCCCTAAAGGGAA	ATCA AT	AAGGCAT T
<i>yraPp</i>	GAGCGACCCAGTTTACCCGCCAT	TGC ACTA	AATACCGATAATGTG	GTCT AA	ACTGCC A
Consensus		GGA ACTT	N15	GTCT AA	

(b)

Fig. 2. (a) Nucleotide sequence alignment of the *S. Typhimurium* σ^E -dependent promoters. The corresponding -10 and -35 regions are depicted in bold. The TSP is in bold and underlined. The *S. Typhimurium* σ^E consensus sequence is shown below the alignment. (b) Determination of the *S. Typhimurium* σ^E consensus sequence. The aligned promoter sequences were analysed using the WebLogo program (<http://weblogo.berkeley.edu/>). The sequences were trimmed at the 3' end to make them all the same length as required by the program. Also, a 'C' residue in the spacer region of the *surAp* promoter was removed to make it the same length as the rest of the promoters. The height of a stack indicates sequence conservation (2 = 100% conservation) and the height of each individual nucleotide within the stack indicates its relative frequency at that position.

Table 2. Function and genetic organization of genes directed by σ^E in *S. Typhimurium* SL1344

IM, Inner membrane; OM, outer membrane; HP, hypothetical protein; asterisks indicate the presence of an internal σ^E -dependent promoter; in all cases putative or known promoters lie to the left of the leftmost genes.

Gene name	Alternative name	Proposed operon structure	Function
Transcriptional factors and regulatory genes			
<i>rpoE</i>	<i>stm2640</i>	<i>rpoE rseA rseB rseC</i>	σ^E /ECF sigma factor; anti-sigma factor; negative regulator; putative regulator
<i>rpoH</i>	<i>stm3568</i>	<i>rpoH</i>	σ^{32}/σ^H /heat-shock factor
<i>rpoD</i>	<i>stm3211</i>	<i>dnaG* rpoD</i>	σ^{70} /principal sigma factor
Primary metabolism functions			
<i>fusA</i>	<i>stm3446</i>	<i>rpsG* fusA tufA</i>	Translation EF-G, EF-Tu
<i>eno</i>	<i>stm2952</i>	<i>pyrG* eno</i>	Enolase (glycolysis)
Periplasmic proteases and folding factors			
<i>htrA</i>	<i>stm0209</i>	<i>htrA</i>	Periplasmic serine protease
<i>ptr</i>	<i>stm2995</i>	<i>recC* ptr recB recD</i>	Periplasmic protease III; exonuclease V β and α subunits
<i>fkpA</i>	<i>stm3453</i>	<i>fkpA</i>	Peptidyl-prolyl- <i>cis-trans</i> -isomerase
<i>surA</i>	<i>stm0092</i>	<i>imp* surA pdxA ksgA apaG apaH</i>	Peptidyl-prolyl- <i>cis-trans</i> -isomerase; pyridoxine biosynthesis; dimethyladenosine transferase; HP
LPS, phospholipids and OM biogenesis			
<i>plsB</i>	<i>stm4235</i>	<i>plsB</i>	Glycerol-3-phosphate acyltransferase
<i>psd</i>	<i>stm4348</i>	<i>yjeQ* psd yjeP</i>	Phosphatidylserine decarboxylase; putative periplasmic binding protein
<i>lpxP</i>	<i>stm2401, ddg</i>	<i>lpxP</i>	Cold-shock-induced palmitoyl transferase
<i>yaeT</i>	<i>stm0224, ecfK</i>	<i>yaeL* yaeT hlpA lpxD</i>	OM biogenesis; histone-like OM protein; lipid A biosynthesis
<i>yfiO</i>	<i>stm2663, ecfD</i>	<i>yfiO</i>	OM lipoprotein involved in OM biogenesis
<i>tolR</i>	<i>stm0746</i>	<i>tolQ* tolR tola tolB pal ybgF</i>	Tol-Pal membrane system, cell envelope integrity, transport through the periplasm
<i>oppA</i>	<i>stm1746</i>	<i>oppA oppB oppC oppD oppF stm1741</i>	Periplasmic oligopeptide transport proteins of ABC family; putative voltage-gated potassium channel
Unknown function			
<i>sbmA</i>	<i>stm0376</i>	<i>sbmA yaiW</i>	Putative ABC superfamily transporter; putative OM lipoprotein
<i>ygiM</i>	<i>stm3203, ecfG</i>	<i>ygiM</i>	Putative IM protein, putative SH3 domain protein,
<i>yggN</i>	<i>stm3107, ecfN</i>	<i>yggN</i>	Putative periplasmic protein
<i>yggT</i>	<i>stm3101</i>	<i>yggS* yggT yggU yggV yggW</i>	Putative integral membrane resistance protein; HP; xanthosine triphosphate pyrophosphatase; oxidase
<i>dedD</i>	<i>stm2364</i>	<i>folC* dedD</i>	Putative membrane protein
<i>yabI</i>	<i>stm0105</i>	<i>yabI</i>	Putative DedA family membrane protein
<i>ycbK</i>	<i>stm0996</i>	<i>ycbK ycbL</i>	Putative OM protein; putative metallo- β -lactamase
<i>yraP</i>	<i>stm3267, ecfH</i>	<i>yraO* yraP</i>	Putative OM lipoprotein
<i>yeaY</i>	<i>stm1819, slp</i>	<i>yeaY</i>	Putative OM lipoprotein
<i>yfeY</i>	<i>stm2447</i>	<i>yfeY</i>	Putative OM lipoprotein
<i>yiaD</i>	<i>stm3645</i>	<i>yiaD</i>	Putative OM lipoprotein
<i>yjfO</i>	<i>stm4379</i>	<i>yjfO ujfN</i>	Putative OM lipoprotein; putative IM protein
<i>yfeK</i>	<i>stm2438</i>	<i>yfeK yfeL</i>	Putative periplasmic protein; penicillin-binding protein, putative membrane carboxypeptidase
<i>yiiD</i>	<i>stm4029</i>	<i>yihZ* yiiD</i>	Putative acetyltransferase
<i>ydcG</i>	<i>stm1622</i>	<i>ydcG</i>	Putative periplasmic glucan biosynthesis protein
<i>stm1250</i>		<i>stm1250* stm1251(agsA)</i>	HP; putative molecular chaperone

biogenesis. Interestingly, *E. coli tolA* and *pal* mutants, which are associated with defects in the bacterial cell envelope, elicit a specific σ^E -mediated ESR that in turn reduces *wzy*-dependent O antigen polymerization (Vines *et al.*, 2005). Increased expression of the mainly periplasmic component of the Tol-Pal system from the internal σ^E -dependent *tolRp* promoter may help *S. Typhimurium* to cope with extracytoplasmic stress by upregulating the production of proteins that are essential for cell envelope integrity. Interestingly, except for *tolR*, all other genes encoding the Tol-Pal system have been found recently to be dependent upon σ^E in *E. coli* (Kabir *et al.*, 2005). This indicates a different σ^E -dependent regulation of this system in the two organisms. Moreover, an *S. Typhimurium tolB* mutant exhibits increased sensitivity to antimicrobial peptides and is less virulent than its wild-type parent as a consequence of the loss of outer membrane stability (Tamayo *et al.*, 2002).

A σ^E -dependent promoter has been located upstream of the *S. Typhimurium oppA* gene. The *oppABCD* operon encodes proteins of the major oligopeptide permease (Opp) that belongs to the ABC transporter superfamily. Opp is the major peptide transport system of enteric bacteria, essential for the uptake of oligopeptides from growth medium and for the uptake and recycling of cell-wall peptides for synthesis of peptidoglycan. In addition to nutrient acquisition, peptide transporters have been shown to play an important role in a diverse array of other functions, including chemotaxis, quorum sensing and conjugation (Detmers *et al.*, 2001; Higgins, 1992). Interestingly, OppA of *E. coli* also has a chaperone-like function, indicating that OppA, together with some other periplasmic substrate-binding proteins, might be involved in protein folding and protection from stress in the periplasm (Richarme & Caldas, 1997). Thus Opp seems to fall into the functional categories of the σ^E regulon and its increased production may be needed under conditions of envelope stress. Expression of the *opp* operon has been suggested to be constitutive in *S. Typhimurium*, but the OppA protein intriguingly accumulates in the periplasm as cells reach stationary phase (Hiles *et al.*, 1987). Analysis of the genomic sequence of *S. Typhimurium* has revealed that, in contrast to *E. coli*, the *S. Typhimurium opp* operon is followed by a potentially cotranscribed gene, *stm1741*, which encodes a putative membrane transport protein similar to voltage-gated ion channels. In *E. coli* two promoters, P2 and P3, directing expression of the *opp* operon have been identified and localized, and there is an additional promoter, P1, which originates in the *IS2* sequence present in some *E. coli* strains (Igarashi *et al.*, 1997). In addition to the σ^E -dependent *oppAp* promoter (Fig. 1), we have identified a σ^E -independent promoter in *S. Typhimurium* which has an identical TSP to the *E. coli* P3 promoter (Fig. 1). Comparison of the *E. coli* and *S. Typhimurium oppA* promoter regions revealed similarity from the ATG codon up to the P3 promoter and in the sequence upstream of the *E. coli* P2 promoter. However, there was no similarity around the *S. Typhimurium*

σ^E -dependent *oppAp* promoter, indicating that the *E. coli oppA* operon is probably not σ^E -regulated.

The *eno* gene encodes the glycolytic enzyme enolase which implicates the σ^E regulon in primary metabolism. Interestingly, enolase has been shown to be a functional part of the membrane-associated RNA degradosome complex essential for mRNA turnover (Bernstein *et al.*, 2004; Carpousis, 2002). Therefore, another proposed role for the σ^E regulon is in specific mRNA turnover during particular conditions of metabolic stress.

Of the remaining *S. Typhimurium* σ^E -dependent genes, 11 (*yggT*, *yggU*, *yggV*, *yggW*, *yjfO*, *yjfN*, *yiaD*, *dedD*, *ycdG*, *yfeK*, *yfeL*) have homologues in *E. coli* and two (*stm1250* and *stm1251*) are specific to *S. Typhimurium*. An *S. Typhimurium* σ^E -dependent promoter has been localized in the coding region of the *yggSTUVW* operon (Table 2). The proposed σ^E -dependent *yggTUVW* genes encode mainly proteins with unknown function (integral membrane protein, putative cytoplasmic protein, putative xanthosine triphosphate pyrophosphatase and putative oxidase). However, in *E. coli*, the *yggV* (*rdgB*) gene has been shown to have a role in DNA repair during DNA replication, most probably due to its xanthosine triphosphate pyrophosphatase activity which helps in avoiding chromosome fragmentation (Bradshaw & Kuzminov, 2003). Thus, this is likely to be a further gene of the σ^E regulon in *S. Typhimurium*, in addition to *recB* and *recD*, with a role in DNA repair and recombination.

Seven *S. Typhimurium* σ^E -dependent genes encode putative outer-membrane lipoproteins that contain a signal sequence typical of bacterial lipoproteins followed by a characteristic lipobox containing a Cys residue which could serve as the lipid attachment site. These include four homologues, YfiO, YraP, YeaY and YfeY, to the recently characterized six σ^E -dependent lipoproteins from *E. coli* (Onufryk *et al.*, 2005) and three other putative outer-membrane lipoproteins YaiW, YjfO and YiaD (Table 2). For the *yjfN*, *dedD* and *yfeK* genes no function could be predicted, although they all encode putative membrane or periplasmic proteins (Table 2), thus indicating a possible function in the cell envelope. The *yfeK* gene is translationally coupled to the *yfeL* gene encoding a putative membrane carboxypeptidase (penicillin-binding protein), probably involved in cell envelope biogenesis. The *ycdG* gene encodes a putative periplasmic glucan biosynthesis protein. It is an orthologue of the recently characterized *ycdG* gene (renamed *mdoD*) encoding the periplasmic OpgD protein involved in the control of the structural glucose backbone of osmoregulated periplasmic glucans (OPG) in *E. coli*. Expression of the *ycdG/mdoD* gene increases during stationary phase (Lequette *et al.*, 2004). Interestingly, mutants defective in OPG synthesis were shown to be highly attenuated or avirulent in several pathogenic bacteria, including *S. Typhimurium*. The OPG seem to be an important component of the cell envelope under extreme environmental conditions and especially during interactions

between pathogenic bacteria with their eukaryotic host (Bohin, 2000). Moreover, OPG have been shown to be essential for resistance to SDS and other anionic detergents (Rajagopal *et al.*, 2003). Hence, all these phenotypes clearly fall into the typical characteristics of the *S. Typhimurium* σ^E regulon.

In the case of the *stm1250* and *stm1251* genes, two *S. Typhimurium* σ^E -dependent promoters, *stm1250p* and *stm1251p*, were localized in close proximity, with TSPs just 394 bp apart. The *stm1250p* promoter is located upstream of *stm1250* which encodes a putative cytoplasmic protein, and the *stm1251p* promoter is located in the *stm1250* coding region, directing expression of the downstream *stm1251* gene which encodes a putative molecular chaperone or small heat-shock protein. Both proteins appear to be specific for *Salmonella* species. However, significant similarity (31–42 % aa identity) to STM1251 has been found with several putative molecular chaperones or small heat-shock proteins of the Hsp20 family from Gram-negative bacteria, including the *E. coli* and *S. Typhimurium* small heat-shock proteins IbpA and IbpB (32 and 31 % identity, respectively). Based on the S1-nuclease mapping analysis, it is clear that both genes, though separated by a 151 bp intergenic region, form an operon and, interestingly, a further heat-shock-inducible promoter having the σ^H consensus sequence has been localized in this intergenic region, directing expression of *stm1251* (data not shown). The product of the *stm1251* gene has been partially characterized recently in *S. Typhimurium*. This gene encodes a novel small heat-shock protein named AgsA (the gene has been renamed *agsA*). Together with the two other small heat-shock proteins, IbpA and IbpB, AgsA has been proved to be an effective chaperone preventing aggregation of non-native cytoplasmic proteins and maintaining them in a state competent for refolding in *S. Typhimurium* at high temperatures (Tomoyasu *et al.*, 2003). Hence, its σ^E -dependence indicates a partial overlap of the cytoplasmic stress response (σ^H -dependent) and ESR (σ^E -dependent) in *S. Typhimurium*. This overlap has also been described recently in *S. Typhimurium*, where activation of σ^E has been shown to enhance expression of the σ^S regulon via σ^H and Hfq during stationary phase, indicating that interactions between alternative sigma factors σ^E , σ^H and σ^S permit the integration of various stress signals to produce coordinated responses (Bang *et al.*, 2005). This study, which was published during the writing of this manuscript, also provided supplementary material detailing *S. Typhimurium* DNA microarray data on the expression profile of σ^E -dependent genes, based on the different stationary-phase mRNA levels in wild-type and *rpoE* mutant strains. Comparison of our σ^E -dependent genes with this transcriptional profiling data revealed that, although 19 genes were at least twofold down-regulated in the *rpoE* mutant, many *S. Typhimurium* σ^E -dependent genes that we have identified were unaffected in the *rpoE* mutant, including such clear σ^E -dependent genes like *rpoH*, *fkpA*, *htrA*, *surA*, *yyaP* and others. This discrepancy may result from the

use of overnight cultures for isolation of RNA from stationary-phase cultures for the DNA microarray experiment. Although σ^E is clearly induced in stationary phase (Miticka *et al.*, 2003; Testerman *et al.*, 2002), in our detailed transcriptional experiment of *S. Typhimurium* σ^E activation during growth in LB medium, we found that σ^E activity peaked at 7 h and then gradually decreased to a rather low level after 14 h (J. Kormanec, unpublished results). Thus, a lower induction ratio of σ^E -dependent genes between wild-type and *rpoE* mutant strains will be seen in long-term stationary-phase cultures, and differences in expression might not be detected.

In conclusion, in *S. Typhimurium*, we identified 34 σ^E -dependent promoters that can potentially direct the expression of 62 genes; among them, 39 orthologues have been previously shown to be σ^E -dependent in *E. coli*. The identified *S. Typhimurium* σ^E -dependent genes fall into similar functional categories, involved mainly in cell-envelope homeostasis, as previously suggested for the *E. coli* σ^E regulon. However, several new functions have emerged, including a role in DNA repair and recombination, and outer-membrane protein assembly. Recent data on alternative *degS*-independent induction of σ^E during carbon starvation in *S. Typhimurium* have suggested that members of the σ^E regulon, in addition to their function in the repair or elimination of damaged cell-envelope proteins, also have additional functions necessary for the adaptation of cells to new environmental conditions (Kenyon *et al.*, 2005). Interestingly, several σ^E -dependent genes have been shown to have a critical role in the virulence in *S. Typhimurium*, thus helping to explain the severe attenuation of *S. Typhimurium* *rpoE* mutants. Further work will be needed to characterize the detail of the biochemical function of these σ^E -dependent genes and their role in the envelope stress response and virulence in *S. Typhimurium*. These experiments are in progress.

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