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The expression of the *rpoE* operon is fine-tuned by the internal *rseAp* promoter in *Salmonella enterica* serovar Typhimurium

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Abstract: Three promoters, located upstream of the *rpoE* gene encoding an extracytoplasmic sigma factor σ^E, direct expression of the *rpoE* operon (*rpoE, rseA, rseB, rseC*) in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). One of them, *rpoEp3,* has been found to be directly recognized by RNA polymerase containing sigma factor σ^E. Using the Northern blot analysis we detected a complex pattern of transcripts indicating an internal promoter in the coding region of the *rpoE* gene. The promoter, *rseAp*, has been located by S1 mapping analysis. Its proposed −10 and −35 elements with 15 intervening nucleotides exhibited high similarity with the consensus sequence of σ^E promoters, suggesting the direct dependence of *rseAp* upon σ^E. Activity of *rseAp* increased towards stationary phase, after heat shock, cold shock, and in the presence of artificially induced *rpoE* expression, the conditions previously shown to activate σ^E-dependent promoters. *In vivo* experiments revealed increase of the *rseAp* activity during growth and confirmed its clear dependence upon σ^E. The proposed role of the internal *rseAp* promoter is to facilitate a feedback control of σ^E level after the envelope stress is removed.

Key words: expression; promoter; regulation; *Salomenella*; sigma factor; stress response.

Abbreviations: LB, Luria-Bertani; TSP, transcription start point.

Introduction

During their life cycle, intracellular, Gram-negative, pathogenic bacteria of the genus *Salmonella* face a wide variety of environmental stresses. The ability of pathogen to cause various types of infections is dependent on its potential to adapt to unfavourable conditions and to avoid the immune response of host organism. One of the mechanisms responsible for ensuring homeostasis in the envelope compartment of the cell, and thus pathogen cells survival, is the envelope stress response, in which sigma factor σ^E plays an important role. The sigma factor σ^E belongs to family of proteins with extracytoplasmic function and it is required for the expression of proteins involved in maintaining the integrity of periplasmic and outer membrane components (Rowley et al. 2006).

The activity of σ^E is tightly regulated. The *Escherichia coli rpoE* gene, encoding the sigma factor σ^E, is essential and located in an operon containing three other genes, *rseA*, *rseB* and *rseC* (Raina et al. 1995; Rouviere et al. 1995; De Las Penas et al. 1997). Under non-stress conditions, σ^E is inhibited by its specific anti-sigma factor, RseA, a membrane-spanning protein whose cytoplasmic domain binds to σ^E (De Las Penas et al. 1997; Missiakas et al. 1997; Ades et al. 1999; Campell et al. 2003), thus preventing its binding to the core RNA polymerase. During the stress, unassembled porin proteins accumulated in periplasm serve as signal to activate the DegS protease to cleave RseA in its periplasmic domain (Walsh et al. 2003; Wilken et al. 2004). Thereafter another protease, RseP, cleaves periplasmically truncated RseA near or within the cytoplasmic membrane to release the RseA cytoplasmic-σ^E complex, and further, cytoplasmic ATP-dependent proteases complete the degradation of RseA, releasing active σ^E (Alba et al. 2002; Flynn et al. 2003; Chaba et al. 2007). After binding to core RNA polymerase, σ^E controls the expression of various genes that are members of the σ^E regulon (Rezuchova et al. 2003; Rhodius et al. 2006).

In contrast to its *E. coli* counterpart, the *rpoE* gene in *Salmonella* Typhimurium is not essential for cell viability, even at high temperature. However, it has been shown to be required for oxidative stress resistance, stationary phase survival, and pathogenicity (Humphreys et al. 1999; Testerman et al. 2002). Although the *S.* Typhimurium *rpoE* operon has a similar organization to its *E. coli* counterpart, its regulation is slightly different. Expression of *S.* Typhimurium *rpoE* is controlled by three promoters, including one, *rpoEp3*, recognized by RNA polymerase containing σ^E (Miticka

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotypes or relevant characteristics[a]	Ref.[b]
Strains		
S. Typhimurium SL1344	*his*, mouse-virulent	[1]
S. Typhimurium GVB311	SL1344 *rpoE*::Km^R	[2]
E. coli XL1Blue	*recA1 endA1 gyrA96 thi-1 hrdR17 supE44 relA1 lac* [F' *proAB lacI*^{q} ZΔ*M15* Tn*10* (Tc^R)]	[3]
Plasmids		
pAC7	Cm^R, low-copy expression vector with P_{DAB} promoter	[4]
pAC-rpoEST4	Cm^R, pAC7 containing *S.* Typhimurium *rpoE* gene under the pBAD control	[5]
pTL61T	Ap^R, low-copy promoterless *lacZ* fusion vector	[6]
pTL61-rseA	Ap^R, pTL61T containing the rseAp promoter DNA fragment	[7]

[a] Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance.
[b] Reference or source: [1], Hoiseth & Stocker (1981); [2] Humphreys et al. (1999); [3], Stratagene; [4], Rezuchova & Kormanec (2001); [5], Miticka et al. (2003); [6], Linn & Pierre (1990); [7], this study.

et al. 2003). We have previously identified 62 genes dependent upon σ^E in *S.* Typhimurium. Products of these genes are required for correct folding of outer membrane proteins and their turnover, phospholipid and lipopolysaccharide biosynthesis, signal transduction, expression of putative inner and outer membrane proteins. Comparison of their σ^E-dependent promoters revealed almost identical consensus sequence (ggAACtt-N_{15}-gTCtaA) to that of *E. coli* (Skovierova et al. 2006).

Functionally related genes are often co-regulated, so that their expression is coordinated in response to the needs of the organism in specific conditions. In prokaryotes these co-regulated genes are often organized in operons. In some cases, however, there may be a need to fine-tune the expression of individual genes in the operon under some specific conditions. This is accomplished by differential regulation of one or more alternative promoters or internal transcription terminators. Therefore, in the present paper, we investigated the expression of the *S.* Typhimurium *rpoE* operon during growth and under several stress conditions.

Material and methods

Bacterial strains, plasmids and culture conditions
The bacterial strains and plasmids used in this study are shown in Table 1. For bacterial growth and transformation, standard conditions and methods were used (Ausubel et al. 1995). Bacteria were grown in Luria-Bertani (LB) medium, or on LB agar plates (Miller 1972). When required, LB medium was supplemented with 100 μg/mL ampicillin, 40 μg/mL chloramphenicol, and 0.2% (w/v) L(+) arabinose. Growth of strains was monitored by measurement of absorbance at 600 nm (OD_{600}). For RNA isolation, the overnight culture of *S.* Typhimurium wild type or *rpoE* mutant strain (GVB311) was diluted 500-fold into 50-mL portions of fresh LB media and grown for 3 h at 37°C to exponential phase (OD_{600} = 0.4) and 7 hours for stationary phase (OD_{600} = 1.7), or in exponential phase cells were exposed to the following stress conditions: 10 min temperature upshift to 50°C (heat shock), 60 min temperature downshift to 10°C (cold shock). For artificial *rpoE* expression, *S.* Typhimurium SL1344 containing pAC-rpoEST4 or pAC7 (negative control) were grown in LB with chloramphenicol to early exponential phase (OD_{600} = 0.2) and expression of *rpoE* was induced with 0.2% (w/v) L(+) arabinose for 2 h. For measuring β-galactosidase activity the overnight culture of the strain *S.* Typhimurium SL1344 and *S.* Typhimurium GVB311 was diluted 500-fold into 50-mL portions of fresh LB media and grown at 37°C for 2 h, 4 h, 6 h, 8 h, and 10 h.

DNA manipulations
DNA manipulations in *E. coli* were done as described by Ausubel et al. (1995). DNA fragments for S1-nuclease mapping were isolated from agarose gel as described by Kormanec (2001). Nucleotide sequencing was performed by the chemical method (Maxam & Gilbert 1980).

Isolation of RNA and S1-nuclease mapping analysis
Total RNA isolation and high-resolution S1 nuclease mapping were performed as described previously (Kormanec 2001). RNA was estimated spectrophotometrically and amount of 40 μg RNA was hybridized to approximately 0.02 pmol of DNA probe labelled at one 5' end with [$\gamma-^{32}$P] ATP (ICN, 4500 Ci mmol/L) and T4 polynucleotide kinase (Promega) as described by Ausubel et al. (1995). The S1-nucelase probe, an 882 bp DNA fragment, was prepared by PCR amplification from the chromosomal DNA of *S.* Typhimurium SL1344 using the 5' end-labelled reverse primer RPE9 (5'-CGTGAACGCACCGTCCCCACC-3') from the end of the *rpoE* coding region and the direct primer RPE7 (5'-GCACGTTATGATTTCGCTGGTGTTGTGC-3').

Nothern blot hybridization analysis
Electrophoresis of total RNA (10 μg) was carried out in 1.2% (w/v) agarose gels in the presence of 2.2 M formaldehyde as described by Ausubel et al. (1995). RNAs were blotted onto Nylon N^+ membrane (Amersham Biosciences) and hybridized at 42°C for 16 h in hybridization solution (50% (w/v) formamide, 5×SSPE, 5×Denhardts, 0.5% SDS, 20 μg/mL fresh denaturated sonicated herring sperm DNA) with digoxigenin-labelled DNA probes prepared according to the manufacture's instruction (Roche). Bands were visualised by exposition to an X-ray film. The following probes were used: probe rpoE (a 555-bp DNA fragment) was prepared by PCR amplification from the chromosomal DNA of *S.* Typhimurium SL1344 using the reverse primer rpoE Bam2 (5'-CCTTTTCCAGGATCCCGCTATCG-3') from the end of the *rpoE* coding region, and the direct primer rpoE Nde2 (5'-GGAGACATTACCTCATATGAGCGAGC-3') from the beginning of the *rpoE* gene; probe rseA (a 344 bp PCR DNA fragment) was prepared using the reverse primer rseA Hind1 (5'-GTGAAACGCACGCAAGCTTACCCATTTGGGTA AGC-3') from the middle part of the*rseA* coding region, and the direct primer rseA Nde1 (5'-GGTATTACATATGCAG AAAGAAAAACTTTCCG-3') from the beginning of the *rseA* gene.

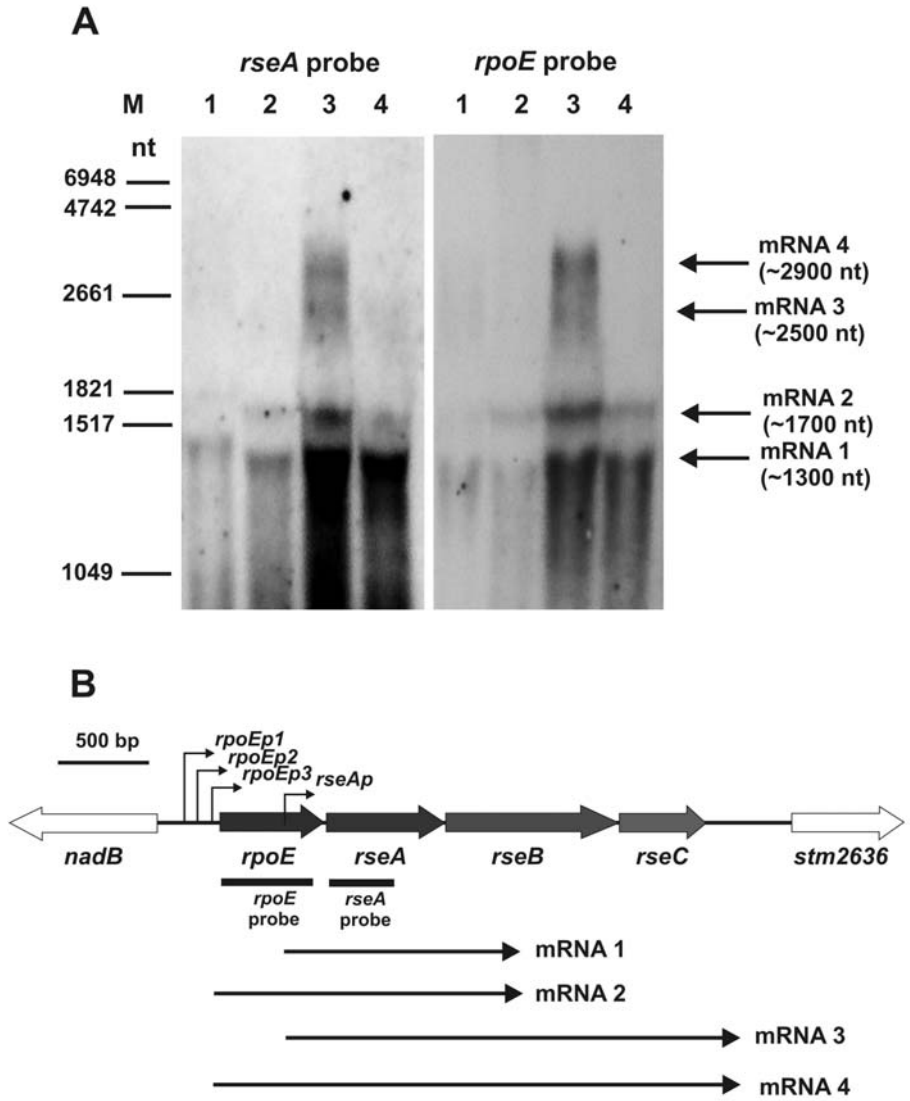

Fig. 1. Northern blot transcription analysis of the *rpoE* operon in *S.* Typhimurium. (A) Total RNA was prepared from the *S.* Typhimurium SL1344 cells grown to exponential phase (lane 1), or exponential phase-grown cells were exposed to 30 min temperature upshift to 45 °C (heat shock) (lane 2), or 1 h at 10 °C (cold shock) (lane 3), or the cells were grown to stationary phase (lane 4). Electrophoresis of total RNA (10 μg) was carried out in 1.2% (w/v) agarose gels in the presence of 2.2 M formaldehyde. After blotting onto Nylon N^+ membrane the blot was hybridized at 42 °C for 16 h in hybridization solution with the digoxigenin-labelled *rpoE* and *rseA* DNA probes (see Materials and methods). The positions of transcripts are indicated by arrows. The sizes of the transcripts were calculated using the digoxigenin-labelled RNA molecular weight marker I (0.3–6.9 kb) (Roche). Positions of standards are indicated on the left. (B) Genetic organization of the *rpoE* operon in *S.* Typhimurium SL1344. The genes are indicated by thick arrows. The thin lines below the map represent the size and putative positions of transcripts obtained by the Northern blot analysis.

Construction of pTL-rseAp and β-galactosidase assay

The plasmid pTL61-rseA containing *S.* Typhimurium *rseAp* promoter cloned upstream the *lacZ* reporter gene in the plasmid pTL61T was prepared by cloning of a 450 bp *Stu*I-*BamH*I fragment from pAC-rpoEST4 (Miticka et al. 2003) into the plasmid pTL-61T (Linn & Pierre 1990) digested by the same enzymes. The resulting plasmid was electroporated into *S.* Typhimurium SL1344 and *S.* Typhimurium GVB311 strains, and β-galastosidae activity was measured during growth in triplicate essentially as described in Miller (1972).

Results

Transcriptional analysis of the rpoE operon by Northern blot hybridization

Previously, expression of the *rpoE* operon (*rpoE*, *rseA*, *rseB*, *rseC*) in *S.* Typhimurium has been found to be driven by three promoters, *rpoEp1*, *rpoEp2* and *rpoEp3*, located upstream of the *rpoE* gene. One of them, the*rpoEp3*, has been found to be strongly induced in stationary phase and by cold shock and verified to be directly recognized by RNA polymerase containing σ^E (Miticka et al. 2003). Northern blot analysis of the *rpoE* operon using total RNA isolated from wild type *S.* Typhimurium SL1344 strain and probes from *rpoE* and *rseA* genes revealed a complex pattern of transcripts (Fig. 1). RNA isolated from the exponential phase (lane 1), after heat-shock (lane 2), after cold shock (lane 3), and from the stationary phase (lane 4) revealed a dominant signal corresponding to mRNA of the approximate length of 1,300 nt, the level of which increased after cold shock conditions and in the

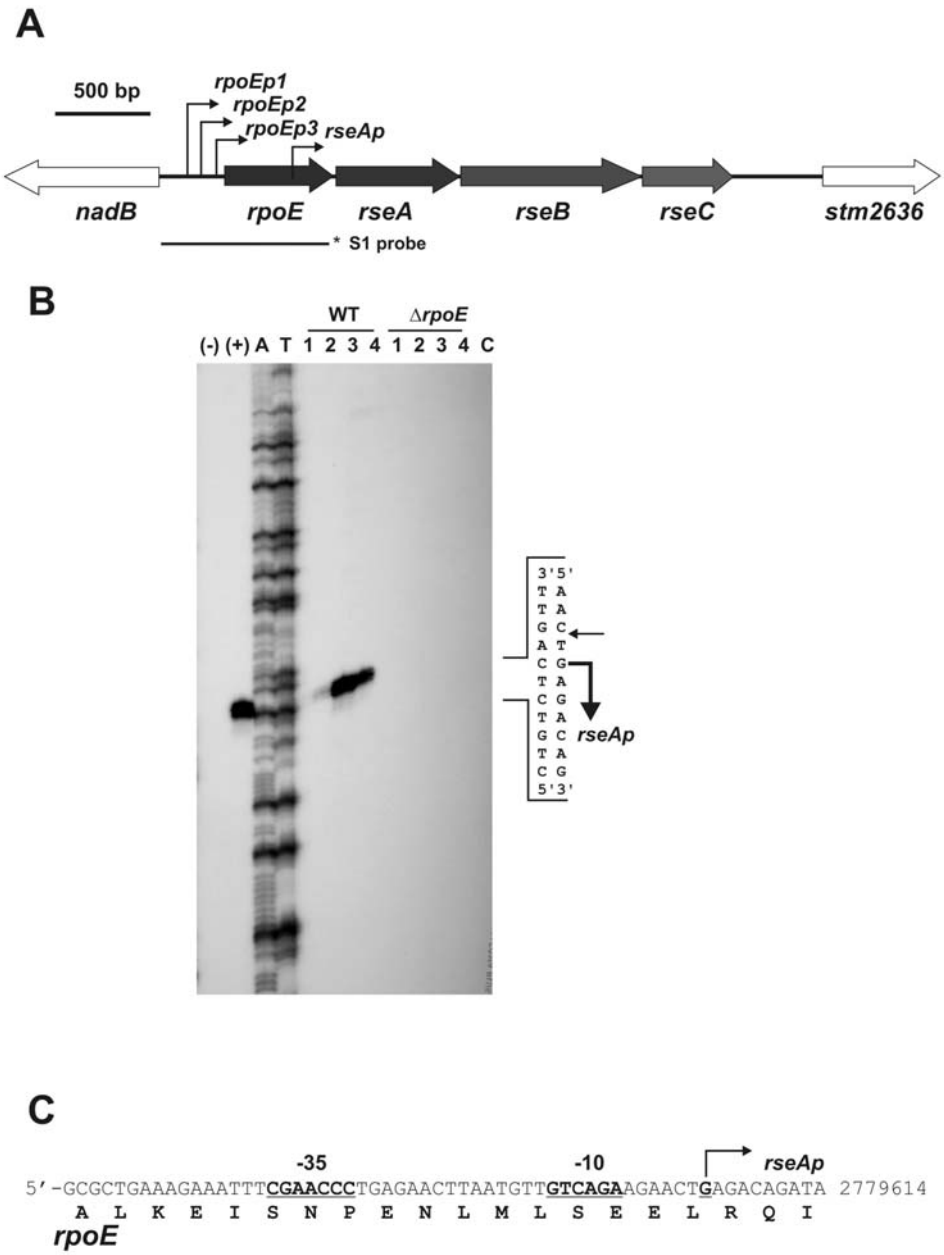

Fig. 2. High-resolution S1-nuclease mapping of the TSP for the *rseAp* promoter in *S.* Typhimurium. (A) Genetic organization of the *rpoE* operon region in *S.* Typhimurium SL1344. The genes are indicated by thick arrows. The thin line below the map represents the DNA fragment (5' end-labelled at the end marked with an asterisk) that was used as a probe in the S1-nuclease mapping experiment. The positions of the promoters are indicated by bent arrows. (B) High-resolution S1-nuclease mapping of the TSP for the *S.* Typhimurium*rseAp*. The 5'-labelled DNA probe hybridized in parallel with 40 μg RNA followed by treatment with 120 U of S1-nuclease (see Material and methods). RNA was isolated from the indicated *S.* Typhimurium strain grown in LB medium. Lane 1, exponential phase (3 h); lane 2, heat shock; lane 3, cold shock; lane 4, stationary phase (7 h); lane (+), RNA was isolated from *S.* Typhimurium SL1344 containing pAC-rpoEST4 grown to early exponential phase and induced for 2 h with 0.2% arabinose; lane (–) control RNA isolated from *S.* Typhimurium SL1344 containing pAC7 grown to early exponential phase and induced for 2 h with 0.2% arabinose; lane C, control *E. coli* tRNA. The RNA-protected DNA fragments were analyzed on DNA sequencing gels together with G+A (lane A) and T+C (lane T) sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert 1980). Thin horizontal arrow indicates the positions of RNA-protected fragment and thick bent vertical arrow indicates the nucleotide corresponding to TSP of the indicated promoter. Before assigning the TSP, 1.5 nt were subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from primer extension products and the chemical sequencing reactions. S1-nuclease mapping experiment was performed twice using independent sets of RNA with similar results. (C) Nucleotide sequence of the *S.* Typhimurium *rseA* promoter region. The protein product corresponding to the *rpoE* gene is given in single-letter amino acid code in the second position of each codon. The TSP of the *rseAp* promoter is indicated by bent arrow. The proposed −10 and −35 boxes of the promoter are in bold characters and underlined. The numbers correspond to the nucleotide positions, which refer to the GenBank/EMBL/DDBJ accession number AE006468.

stationary phase that are the conditions inducing σ^{E}-dependent promoters (Miticka et al. 2003). However, there was another mRNA of the approximate length of 1,700 nt, the level of which was similarly induced. At cold shock conditions, in addition to these mRNA species, there were two additional signals correspond-

ing to mRNA of the approximate lengths of 2,500 and 2,900 nt. Based on the *S.* Typhimurium *rpoE* operon structure (Fig. 1B), the 1,700 nt mRNA might initiate from the *rpoEp3* promoter and cover the *rpoE* and *rseA* genes (thus terminating in the *rseB* gene). Supposing the similar terminating (or transcript processing) site, the smaller mRNA (1,300 nt) thus should initiate from an *rpoE* internal promoter. Actually, we have identified and characterized this *rseAp* promoter in this region (see below). Both transcripts thus would terminate downstream of the *rseA* gene, in the *rseB* coding region (Fig. 1B). However, computational search has not identified any putative terminator-like sequence in this region. Considering the *S.* Typhimurium *rpoE* operon structure (Fig. 1B), the cold-shock-induced 2,500 nt and 2,900 nt mRNA species may similarly initiate from the *rseAp* and *rpoEp3* promoters, respectively, and terminate downstream of the last gene in the operon, *rseC*. However, a computational search have again identified no putative terminator-like sequence in this region.

Location of the rpoE-internal rseAp promoter by S1-nuclease mapping

To confirm the Northern blot analysis results and to localize the *rseAp* promoter, high-resolution S1-nuclease mapping was performed using the 5'-labelled S1 probe (Fig. 2A). RNA was isolated from *S.* Typhimurium SL1344 and its isogenic *rpoE* mutant *S.* Typhimurium GVB311 (Humphreys et al. 1999) grown to different growth phases, after stresses, and after artificially-induced *rpoE* in the wild-type strain. Results shown in Fig. 2B revealed presence of a single promoter, *rseAp*, that was clearly dependent upon *rpoE* (the transcript was missing in the *rpoE* mutant). Its activity partially increased after heat shock (lane 2) and dramatically increased towards stationary phase (lane 4) and after cold shock (lane 3), the conditions previously characterized to most increase activity of σ^E-dependent promoters (Miticka et al. 2003). When RNA was isolated from *S.* Typhimurium SL1344 containing pACrpoEST4 grown to exponential phase and induced for 2 h with 0.2% arabinose (after artificially induced *rpoE*), the activity of *rseAp* was significantly induced (lane +), but no band was observed when empty vector was used (lane −). We localized the transcription start point (TSP) of the *rseAp* promoter in the *rpoE* coding region (Fig. 2C). The proposed −10 and −35 elements, GGAACCC-N_{15}-GTCAGA, exhibited a high similarity with the consensus sequence of the σ^E promoters, GGAACTT-N_{15}-GTCAAA (Skovierova et al. 2006), with only one mismatch in the −10 element and two mismatches in the −35 element. However, all the critical nucleotides of σ^E-dependent promoters (Miticka et al. 2004) were present in the *rseAp* promoter. The *rseAp* promoter in the similar position was previously identified in the *rpoE* operon of *E. coli* by transcription profiling in wild-type strain and the strain overexpressing *rpoE* (Rhodius et al. 2006).

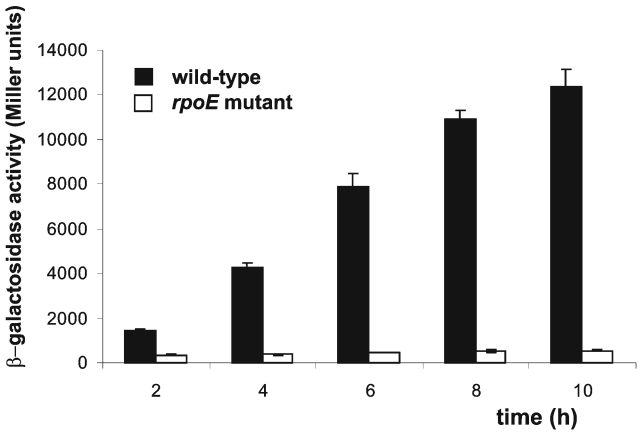

Fig. 3 Transcriptional activity of the *rseAp* promoter during growth in *S.* Typhimurium. β-Galactosidase activity of an *rseAp-lacZ* transcriptional fusion in the wild-type *S.* Typhimurium SL1344 and the *rpoE*-mutant, *S.*Typhimurium GVB311, strains containing the plasmid pTL-rseAp was measured at the indicated time points during the growth in LB medium. The time points represent the following growth phases: 2 and 4 h, exponential phase; 6 h, end of the exponential phase; 8 and 10 h, stationary phase. Each point represents the mean of three assays, and error bar indicates the standard deviation from the mean.

Verification of the promoter in vivo after fusion to the lacZ reporter gene

Expression of the *S.* Typhimurium *rseAp* promoter was investigated *in vivo* in *S.* Typhimurium as an *rseAp-lacZ* reporter fusion after cloning of the promoter-encompassing DNA fragment into the promoter-probe plasmid pTL61T. The resulting plasmid pTL61-rseA was electroporated into the wild-type *S.* Typhimurium SL1344 and its isogenic *rpoE* mutant, *S.* Typhimurium GVB311, and β-galactosidase activity was measured in triplicate during growth in LB medium. As shown in Figure 3, the *in vivo* activity of the *rseAp* promoter strongly increased towards the stationary phase in the wild type strain (6 h represents the entry into stationary phase). There was almost no activity found in the *rpoE* mutant strain GVB311 indicating a clear dependence of *rseAp* upon σ^E. These results are consistent with our *in vitro* findings and our previous *in vivo* data on σ^E-dependent promoters in *S.* Typhimurium (Miticka et al. 2003).

Discussion

Transcriptional regulation constitutes an important step in the control of gene expression. In bacteria, functionally related genes are often organized in operons. Individual mRNAs contain several genes with related function, thus coordinating the synthesis of the proteins encoded by them. However, there might be circumstances when an organism may need the products of the genes in an operon differentially. Therefore, some flexibility may be necessary to escape from the coordinated expression in an operon in some specific conditions. This may be insured by an alternative regulation of the genes in the operon by means of promoters or

transcription terminators within an operon to allow for differential expression of genes in the operon.

We have previously identified three promoters upstream of the *S.* Typhimurium*rpoE* operon (Miticka et al. 2003). The *rpoEp1* and *rpoEp2* promoters were constitutively expressed during growth and their proposed role is to provide basal level of *rpoE* operon transcription at non-induced conditions at exponential phase. The *rpoEp3* promoter was weakly expressed in exponential phase, but it was greatly induced in the stationary phase and after cold shock, and to less extent also some other stress conditions. Its activity was dependent upon σ^E. Its proposed role is therefore to amplify transcription of the *rpoE* operon at the envelope stress conditions to ensure fast response to these detrimental conditions. However, it the present work we identified and characterized the other σ^E-dependent promoter, *rseAp*, located in the *rpoE* gene, thus directed transcription only of the three genes, *rseA*, *rseB* and *rseC*. RseA as an anti-sigma factor of σ^E plays a critical role in negative regulation of σ^E during unstressed conditions. A periplasmic protein RseB is not so critical and only fine-tunes the negative regulation of RseA (De Las Penas et al. 1997; Missiakas et al. 1997). Even though the role of RseC is not clear yet, it may act in a subtle but positive modulating effect on σ^E (Missiakas et al. 1997), or function in the reducing system of the superoxide sensor SoxR, which is involved in protection of cells against superoxide and nitric oxide (Koo et al. 2003). The coordinate transcription of genes composing the *rpoE* operon is thus necessary for its precise regulation. The results showing that the synthesis of RseA is driven from two stress-induced σ^E-dependent promoters, *rpoEp3* and *rseAp*, indicate that higher production of RseA is needed at stress conditions, since after relieving free σ^E from the RseA-σ^E complex, the RseA is permanently degraded by the proteolytic cascade of two membrane proteases, DegS and RseP, followed by cytoplasmic ClpXP protease complex (Alba et al. 2002; Flynn et al. 2003; Chaba et al. 2007). This organization of gene expression of the *rpoE* operon likely ensures the sufficient level of RseA for the rapid down-regulation of the σ^E activity following the decrease of accumulation of unfolded outer membrane proteins in periplasm when the envelope stress is omissible. In fact, the ratio of σ^E and RseA is almost constant during the stress conditions, and after shutoff of stress the activity of σ^E is strongly repressed and then slowly returns to the level of unstressed conditions (Ades et al. 2003).

The results of Northern blot analysis of the *rpoE* operon in *S.* Typhimurium are consistent with the data of the finding of complex regulation by two σ^E-dependent stress-induced promoters. However, the pattern of transcripts is more complex (Fig. 1), and indicates their premature termination or processing in the *rseB* coding region. Absence of a putative terminator-like sequence in this region favours the mRNA processing hypothesis, however, we cannot rule out a premature transcription termination as a mechanism for production of this transcript pattern. Stabilization of RNA by low temperature during cold shock could be the reason for detection of larger non-processed transcripts (2,500 nt, 2,900 nt). Further experiments are therefore necessary to explain this processing more clearly. These experiments are in progress.

In conclusion, using the S1 mapping analysis we identified the internal *rpoE* operon promoter, *rseAp*, in *S.* Typhimurium. Suggested −10 and −35 regions of the *rseAp* exhibited the high similarity with the σ^E consensus sequence. Our results revealed the increase of the *rseAp* activity towards stationary phase, after heat shock, cold shock and in the presence of artificially induced *rpoE* expression, the conditions previously shown for *rpoE* induction. Dependence of *rseAp* upon *rpoE* was confirmed by *in vivo* experiments. A pattern of transcripts detected by Northern blot analysis indicates the complex regulation of the genes composing the *rpoE* operon in *S.* Typhimurium.

Acknowledgements

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