Sigma E controls biogenesis of the antisense RNA MicA

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ABSTRACT

Adaptation stress responses in the Gram-negative bacterium Escherichia coli and its relatives involve a growing list of small regulatory RNAs (sRNAs). Previous work by us and others showed that the antisense RNA MicA downregulates the synthesis of the outer membrane protein OmpA upon entry into stationary phase. This regulation is Hfq-dependent and occurs by MicA-dependent translational inhibition which facilitates mRNA decay. In this article, we investigate the transcriptional regulation of the micA gene. Induction of MicA is dependent on the alarmone ppGpp, suggestive of alternative σ factor involvement, yet MicA accumulates in the absence of the general stress/stationary phase σE. We identified stress conditions that induce high MicA levels even during exponential growth—a phase in which MicA levels are low (ethanol, hyperosmolarity and heat shock). Such treatments are sensed as envelope stress, upon which the extracytoplasmic sigma factor σE is activated. The strict dependence of micA transcription on σE is supported by three observations. Induced overexpression of σE increases micA transcription, an Δpoe mutant displays undetectable MicA levels and the micA promoter has the consensus σE signature. Thus, MicA is part of the σE regulon and downregulates its target gene, ompA, probably to alleviate membrane stress.

INTRODUCTION

Bacteria meet adverse environmental conditions by rapid adaptive changes in gene expression patterns, thereby mounting appropriate responses. This most often involves transcriptional regulation, by repressors or activators, often under the control of two-component regulatory systems, and/or by alternative sigma (σ) factors.

In addition, a second level of posttranscriptional control is frequently involved. In Escherichia coli, a growing number of small non-coding RNAs (sRNAs) have been implicated in the regulation of stress responses and virulence traits (1–3). Many of these are conserved in enteric relatives, and quite a few sRNAs have since been identified in other bacteria as well.

Most of the sRNAs are antisense RNAs that inhibit (or, less frequently, activate) the translation of target mRNAs or promote their degradation. The ubiquitous RNA-binding protein Hfq (4) is often required for regulatory activity, though its mechanism of action is not yet fully understood.

In contrast to the cis-encoded antisense RNAs in plasmids, most chromosomally encoded sRNAs are trans-encoded (5). Their genes do not overlap target genes, and thus complementarity to the target mRNA is limited and often non-contiguous. Therefore, the location of an sRNA gene does not automatically identify the target gene. Assigning functions for new sRNAs has been based on screening for downstream effects in strains lacking the sRNA, or overexpressing it. Microarray analyses, two-dimensional protein gels, phenotypic tests and bioinformatics-aided complementarity searches have resulted in the identification of targets (6).

Conceptually, sRNAs are expected to be under appropriate transcriptional control, so that their induction matches requirements for their regulatory activity. This appears to be borne out by observations: for instance, RyhB is under control of the Fur repressor. When iron concentration is low, Fur repression of the ryhB promoter is abolished, and synthesized RyhB inhibits the synthesis of several iron-binding proteins (7). A similar logic guides the expression characteristics of several other sRNAs.

Many sRNAs are upregulated immediately before or upon entry into stationary phase (8,9). This stress response in E. coli has been studied extensively and is characterized by major physiological changes arising from orchestrated alterations in gene expression (10), the majority of which are dependent on the stress/stationary phase σE. This transcription factor, in turn, is under
intricate control involving several environmental signals that converge on the expression of its gene, the translation or stability of its mRNA and the activity/stability of the protein itself (11). Among these regulators are two antisense sRNAs, DsrA and RprA, that are induced by different signals and enhance translation of the rpoS mRNA (12). Transcriptome and proteome analyses have been used to chart the σ^E regulon, which includes transcriptional regulators important for the scavenging of nutrients, such as Crp and the two-component Ntr and Pho systems, and many genes whose products help in adaptation to stationary phase. Recently, microarray data have contributed to the understanding of the transcriptomes of other σ factors, such as the heatshock σ^H (13) and the extracytoplasmic stress σ^E (14). Interestingly, σ^H responds not only to heat stress, but is itself transcriptionally upregulated in a σ^E-dependent manner (15), perhaps indicative of some regulatory ‘hierarchy’.

The extracytoplasmic function (ECF) σ^E is specifically induced in response to conditions that culminate in changes within the periplasm (16). Its intracellular levels are high, almost as high as those of the housekeeping σ^0 (17). σ^E is sequestered at the cytoplasmic membrane by an anti-σ factor, RseA. Upon accumulation of misfolded proteins in the periplasm, ethanol exposure, heat shock and hyperosmotic stress, the distress signals are perceived by the protease DegS. Successive cleavages of RseA by DegS and RseP, a second protease, release the active σ^E into the cytoplasm to compete for RNA polymerase. More than 80 genes are induced in response to σ^E upregulation (14,18,19). σ^E is an essential protein in E. coli (20), which likely testifies to the importance of the periplasmic and outer membrane compartments for many life processes.

We and others have studied the sRNA MicA (earlier: SraD (8)). MicA accumulates upon entry into stationary phase and, aided by Hfq, downregulates the translation of the outer membrane protein OmpA, concomitantly inducing the degradation of the ompA mRNA (21,22). The signals and effectors that control transcription and accumulation of MicA were unknown. In this work, we investigated the transcriptional regulation of MicA under a variety of stress conditions. We find that transcription of MicA is strictly dependent on σ^E, making it the first sRNA in E. coli shown to be an exclusive member of the σ^E regulon.

**MATERIALS AND METHODS**

**Promoter sequence alignment**

Selected micA upstream sequences from several enterobacterial genomes (gamma proteobacteriaceae subdivision) were aligned. We aligned the sequence ranging from the known E. coli micA gene —55 to +4 sequence (+1 being the transcription start site (8; K.U., unpublished)) with corresponding regions in related genomes. Alignment to the consensus rpoE recognition module was based on previous research (14,19). Sequences were obtained from the NCBI database, and BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and accession numbers indicated are listed in the Figure 3 legend.

**Bacterial strains and plasmids**

Bacterial strains and plasmids are listed in Table 1. The E. coli strain MC4100relA^+ was used as wildtype unless otherwise stated. To construct plasmid p30-luc, we cleaved out the promoter region of pZE12-luc (23) with the restriction enzymes EcoRI and XhoI. The micA promoter region was amplified using primers KU30-LmicA and KU32-pMic, and fragment was cloned into the XhoI site of pZE12-luc plasmid (23) with the EcoRI site underlined. The resulting 106 bp DNA fragment containing micA (—85) to (—1) flanked by XhoI and EcoRI sites was digested, purified and ligated into the linearized plasmid. Plasmid p30-luc thus carried the luc gene under transcriptional control of micA’s —85 to —1 region. A promoterless control plasmid (pZE12b-luc) was constructed by treating the EcoRI- and XhoI-cleaved

**Table 1.** Strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| Strain           |             |                     |
| MC4100relA      | araD139, Δ(argF-lac)205, flb-5301, pstF25, rpsL150, deoC1, relA1 | (21) |
| MC4100relA^- | relA^- derivative of MC4100 | (21) |
| MC4100relA^- rpoS::Tn10 | Sigma S deficient derivative of MC4100relA^- | Shoshy Altuvia |
| MC4100 relA3spoT | ppGpp-null derivative of MC4100 | Thomas Nyström |
| MC4100 relA1 cya^- | cAMP^- deficient MC4100 | B.E. Uhlin |
| CAG16037 | araD Δ[ara-les]-7097, Δ(codB-lacI), galK16, galE15, mcrA0, relA1, rpsL150, spoT1, mcrB999, hsdR2 [φ[priH 85]::lacZ] | (40) |
| CAG16037 ΔrpoE | araD Δ[ara-les]-7097, Δ(codB-lacI), galK16, galE15, mcrA0, relA1, rpsL150, spoT1, mcrB999, hsdR2 [φ[priH 85]::lacZ] | (41) |
| CAG22216 | CAG16037 ΔrpoE | Invitrogen |
| LMG194 | F^- ΔlacX74, galE, thi, rpsL, ΔphoA, Δara714, leu::Tn10 | This work |
| GW 986 | LMG194 (pACYC184) | This work |
| GW 989 | LMG194 (pAC-rpoE4) | This work |
| GW 962 | MC4100relA^- (p30-luc) | This work |
| GW 982 | MC4100 relA^- (pZE12b-luc) | This work |
| Plasmid |             |                     |
| pAC-rpoE4 | rpoE gene with arabinose-responsive promoter | (19) |
| pZE12-luc | p15A plasmid carrying pLac-luc gene | (23) |
| pZE12b-luc | pZE12-luc derivative lacking the pLac promoter | This work |
| p30-luc | micA promoter (—85 to +1) transcriptionally fused to luc gene | This work |
pZE12-luc with Mung bean nuclease prior to re-ligation. The arabinose-controlled σ^E overexpression plasmid pAC-rpoE4 was a gift from Dr. Jan Kormanec, Bratislava, Slovak Republic.

**Media and growth conditions**

Cells were grown aerobically at 37°C in LB broth (LB), unless otherwise specified. Bacterial growth was monitored by measuring OD_{600}. When required, antibiotics were added at 50–100 μg/ml (ampicillin) or 30 μg/ml (chloramphenicol). Where necessary, medium was supplemented with thiamine (0.2%). In *vivo* labeling experiments were carried out with cultures growing in complete M9 minimal medium lacking the amino acids cysteine and methionine and containing 0.4% glycerol as carbon source. Induction of gene overexpression was carried out in the presence of 10 mM L-arabinose.

**Bacterial stress challenge**

*E. coli* strain MC4100relA+ was diluted 250-fold from an overnight culture into pre-warmed LB medium and allowed to grow aerobically at 37°C to an OD_{600} of ~0.3. The culture was split, centrifuged at 8000 g, washed with 0.9% [w/v] NaCl and recentrifuged. Cell pellets were resuspended in equal volumes of pre-warmed stress medium, and further grown for 30 min. Stress media were prepared with LB, supplemented with either 10% EtOH (ethanol shock), 2% bile salts (bile), 0.5 M NaCl (hypoosmotic shock) or 20% sucrose (hyper-osmolarity). For alkaline stress, LB was adjusted to pH 9 with 4 M NaOH. For heat shock, an overnight culture was treated as above and grown at 30°C. Growth with vigorous shaking was continued for 30 min.

**rpoE overexpression**

Plasmid pAC-rpoE4 was transformed into the arabinose-insensitive LMG194 strain. Overnight cultures in LB medium supplemented with chloramphenicol at 37°C were diluted 250-fold into prewarmed medium. At OD_{600} ~0.2, the culture was divided in two, centrifugated and pellets washed once with 0.9% NaCl. Bacteria were resuspended in prewarmed LB with or without arabinose (10 mM). Samples were taken for RNA and protein analysis at the time points indicated in Figure 4.

**Northern blot analyses**

Cell growth was stopped in 0.2 ml of RNA stop solution (5% phenol, 95% EtOH), and samples were pelleted and frozen in liquid nitrogen. Total RNA was extracted using the hot acid–phenol method essentially as described (24). Total RNA was treated with RQ1 DNase (Promega), extracted with phenol, chloroform, and precipitated in ethanol at ~20°C. RNA pellets were washed in 75% ethanol, dried at room temperature and dissolved in RNAse-free water. One ml of RNA loading buffer (95% [v/v] formamide, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol, 0.025% [w/v] SDS, 5 mM EDTA pH 8.0) was added. Electrophoresis of total RNA (~10 μg) was carried out on 5 or 6% polyacrylamide/7 M urea gels. Gels were electroblotted (Bio-Rad Trans-Blot cell) onto Nylon N+ membranes (GE Healthcare) and probed in modified Church and Gilbert hybridization buffer. Probing with DNA oligodeoxyribonucleotides was carried out at 42°C or with riboprobes at 65°C. Bands were visualized with a PhosphorImager, model 400S (Molecular Dynamics), and quantitated using ImageQuant software, version 4.2a (Molecular Dynamics).

**Probe generation**

DNA probes were generated by 5’-end-labeling of RNA-specific oligodeoxyribonucleotides with a molar excess of γ-32P-ATP. The MicA riboprobe was generated by *in vitro* transcription, as described (21). All probes were purified on G50 Microspin columns (GE Healthcare).

**Western blot analyses**

Cultures were stopped by addition of chloramphenicol to a final concentration of 200 μg/ml and mixing. Cells were pelleted, resuspended and boiled in SDSBME [0.3% (w/v) SDS, 0.2 M DTT, 0.028 M Tris-HCl, 0.022 M Trizma base] for 2 min. Equal amounts of protein were analyzed on 10% SDS-PAGE gels. Proteins were blotted onto PVDF membranes, and membranes blocked at 4°C overnight with BLOTTO (5% (w/v) dried milk dissolved in PBS containing 0.5% (w/v) Tween20). Blots were incubated in BLOTTO with rabbit monoclonal antibodies targeting σ^E or σ^70 (Neocline). Dilutions of antibodies were 1:1000 (σ^70) or 1:10000 (σ^E). The hybridized blots were incubated with HRP-conjugated anti-rabbit IgG (1:10000) as above, prior to visualization using the ECL Plus (GE Healthcare) Western blotting detection kit. Blots were exposed to x-ray film and developed on an OPTIMAX 1170-L-0000 x-ray film processor (Protec GmbH). Band intensities were measured with Quantity One software (Bio-Rad). Monoclonal antibody to σ^70 was a gift from Thomas Nyström, Göteborg University, Sweden.

**Transcription rate assay by luciferase reporter**

Aliquots of 1 ml were taken from growing cultures, quenched in chloramphenicol (200 μg/ml) and snap-frozen in liquid nitrogen. Samples were lysed and assayed for luciferase activity according to the manufacturer’s protocol (Luciferase Assay Kit, SIGMA) on a Bio-orbit 1253 luminometer (Bio-orbit Oy). Background luminescence was obtained from cells carrying the transcriptionally inactive control plasmid (pZE12b-luc) under the same conditions.

**Immunoprecipitation assay**

An overnight culture of LMG194::pAC-rpoE4 was diluted 500-fold in M9 minimal medium containing all amino acids except methionine and cysteine. At OD_{600} ~ 0.3, the culture was split, one half induced with arabinose, the
other mock-induced. After 20 min, samples were taken to control for MicA induction (not shown). Aliquots of the same cultures (2 ml) were then pulse-labeled with 300 μCi of $^{35}$S-Met (>37 TBq/ mmol) for 50 min, shaking vigorously. Cultures were chased with a thousand fold molar excess of non-radioactive Met for 2 min and then quenched in chloramphenicol (200 μg/ml). Cell lysates (prepared as described above) were precleared with Protein A Sepharose equilibrated in Immunoprecipitation (IP) Lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) NP-40] for 1 h on ice. Equal volumes of precleared lysates were incubated overnight with either anti-σ$^E$ monoclonal antibody or anti-OmpA polyclonal antibody at 4°C on a rotating platform. Unbound proteins were removed by five consecutive IP lysis buffer washes. Laemmli loading buffer was added to the sepharose, and elution of proteins was carried out by vortexing and heating to 95°C for 10 min. Aliquots were separated on 10% SDS-PAGE/3.5 M urea gels. Gels were dried and exposed to a PhosphorImager screen, images scanned and band intensities quantitated.

RESULTS

Stress conditions affect the accumulation of MicA RNA

The intracellular levels of MicA increase substantially upon entry into stationary phase during E. coli growth under laboratory conditions. Adaptation to stationary phase growth involves the major stress response sigma factor, σ$^S$, whose synthesis and activity is in turn induced by a number of different stress signals—among these are acid stress (25) and osmotic shock (26). Hence, we tested which environmental stresses would upregulate the levels of MicA, and whether σ$^S$ was required.

In a first set of experiments, we monitored the levels of MicA in response to stress challenge during logarithmic growth, the phase at which intracellular MicA (and σ$^S$) concentrations are normally low. The E. coli strain MC4100ΔrelA$^+$ was subjected to stress treatments as described in Materials and Methods. After 30 min, total RNA was extracted for Northern blot analysis. Figure 1A shows that loading-corrected MicA levels (5S rRNA as control) were significantly increased, compared to mock treatment, upon heat shock (~threefold; cf. lane 30–30 to 30–42°C), ethanol stress (~sevenfold; ethanol), and growth at hypoosmolarity (~ninefold; NaCl). Only a marginal increase (~twofold) was observed when E. coli was exposed to hyperosmolarity (lane Sucrose) and bile salts failed to induce. The increased levels of MicA after ethanol treatment were accompanied by a significant processing/partial degradation of this RNA. Stresses such as osmotic shock (whether hyperosmotic or hypoosmotic) are known to induce the rpoS regulon (26). Thus, we tested whether the observed patterns of MicA upregulation (Figure 1A) required σ$^S$. An isogenic strain pair, rpoS$^+$/ΔrpoS, was subjected to selected stress conditions, and intracellular MicA levels were assessed by Northern blot as before. The strong induction of MicA accumulation by, in particular, NaCl, is σ$^S$-independent (Figure 1B). Though a contribution of σ$^S$ is not ruled out by these experiments, the experiment indicates that another σ factor is responsible for MicA transcription. Note that in early exponential phase, MicA levels were higher (~threefold) in a ΔrpoS than in an rpoS$^+$ background.

E. coli mutant strains exhibit aberrant MicA levels

Earlier attempts to transcribe the micA gene in vitro by RNA polymerase holoenzyme (containing σ$^S$) had been unsuccessful (8; K.U., unpublished). This suggests that an alternative σ factor is required for transcription. σ$^S$ was tentatively ruled out by the experiment in Figure 1B. Sigma factor competition, resulting in the exchange of the housekeeping σ$^S$ for alternative σ factors, is thought to be dependent on the nutritional stress signal ppGpp (27). Thus, we monitored the growth-phase-dependent
accumulation of MicA in strains carrying mutations that decrease or abolish ppGpp synthesis (spoT, relA1), and strains that are either rpoS – or cyaA – (adenyl cyclase). The relA – strain showed stationary phase induction as previously reported (Figure 2A and B, left panels). Induction was only slightly affected in an isogenic relA1 strain (Figure 2B, middle panel), but severely impaired when both ppGpp synthetases, SpoT and RelA, were mutated (Figure 2A, middle panel). More specifically, we consistently noted a shift in the timing of MicA induction in the relA1 mutant strain, which we attribute to SpoT-dependent ppGpp production (Figure 2B; cf. lanes 7 and 8 to lanes 3 and 4). MicA began to accumulate already in late exponential phase (lane 7), but stationary phase levels were identical to wild-type (Figure 2B; cf. lanes 8 and 9 to lanes 4 and 5). A ppGpp requirement for growth-phase-dependent upregulation of the sRNA is consistent with transcription being dependent on an alternative σ factor, although some σ30 promoters are known to be affected by this alarmone as well. Compared to its wild-type counterpart, the rpoS strain mutant showed, however, only a modest reduction of ~40% (Figure 2B, right panel). Thus, σ3 cannot be the main factor driving MicA transcription (Figures 1B and 2B), even though a minor contribution may be due to redundancy of σ-factor recognition during transcription initiation (28,29). It is worth noting that accumulation of MicA could have been caused by a decrease in its decay rate rather than an increase in transcription rate. Rifampicin run-out experiments, however, failed to show significant differences in MicA half-life in early and late growth (data not shown).

Interestingly, the cyaA mutant strain (deficient in cAMP production) showed an entirely different expression pattern. It produced >ten fold higher levels of MicA in early exponential phase (Figure 2A, right panel, OD600 0.3) compared to wild-type (lane 2). MicA levels in the cyA mutant remained high throughout growth until OD600 ~ 2.4. At this point in the growth curve, the MicA level of the wild-type strain approached that of the cyA strain. The possible involvement of catabolite repression, which is relieved in a cyA mutant strain, was tested by comparisons of MicA levels in both mutant and wild-type cells in the presence or absence of glucose. Glucose did not affect MicA levels differentially in the two strains (data not shown).

The micA upstream region is highly conserved and has a σE recognition signature

The experiments that indicated membrane stresses (ethanol and osmolarity changes) to be inducers of MicA accumulation, and the functional connection of MicA as a regulator of an outer-membrane protein, suggested σE to be responsible for MicA transcription. We analyzed the promoter region of the micA gene in E. coli and several enterobacteria in which homologs could be identified. Since the +1 transcription start site had been mapped (8, data not shown), the putative promoters and their upstream regions could be compared to each other, and to the σE consensus binding motifs identified by the Gross and Kormanec labs (14,19). The BLAST-aligned putative micA promoter sequences show all characteristics of the rpoE promoter sequence, which is known to be transcribed by σE. Strong sequence conservation within the −35, and −10 boxes was observed in micA promoters of E. coli and several relatives (Figure 3). Full sequence conservation was seen in the ‘AA’ tract of the −35 element, which was recently postulated to be essential for σE recognition (30). Spacer and discriminator regions are of median size at 16 and 6bp, respectively. The −10 box contains an invariable C-residue found in σE-controlled promoters, and the region upstream of the −35 box comprises a conserved AT-rich element (14). Interestingly, this element differs in Serratia marcescens, a bacterial species that does not show growth-phase-dependent MicA upregulation (21). The micA promoter (without upstream sequences) is 85% (−35 box) and 100% identical (−10 box) in sequence to the P3 promoter of rpoH—a promoter that is exclusively transcribed by σE.

Overexpression of σE results in increased MicA accumulation

In order to ascertain σE-driven transcription of MicA, we tested whether overexpression of the ECF would upregulate micA expression. E. coli strain LMG194 (Table 1), carrying a multicopy plasmid bearing an arabinose-inducible copy of the rpoE gene, was either induced or mock-treated. Samples were taken for protein and RNA analysis at specific time points after induction. Figure 4A shows a Northern blot probed with a MicA-specific riboprobe or a 5S rRNA-specific oligo probe as a loading control. Arabinose addition resulted in a rapid increase in MicA, already visible at the 5-min timepoint. MicA levels were maximal within 10 min of induction, reaching a plateau ~5 fold higher than in the uninduced samples. Figure 4B shows a Western blot analysis of total protein lysate from the same time points confirming...
the induction of $\sigma^{E}$, whereas the level of the housekeeping $\sigma^{70}$ was essentially unaffected.

The effect of $rpoE$ overexpression on the micA gene is transcriptional

The experiment in Figure 4 suggests that $\sigma^{E}$ is responsible for initiation at the micA promoter. If true, a micA promoter fused to a reporter gene should show growth-phase-dependent upregulation. Plasmid p30-luc carries the micA promoter in front of a luc (luciferase) reporter gene. The control plasmid pZE12b-luc is promoterless. E. coli cells harboring either of the two plasmids were diluted after overnight growth and allowed to grow. Samples were withdrawn for determination of luciferase activity at the OD$_{600}$ values indicated in Figure 5A. Background luciferase activity from the control strain (promoterless plasmid) was subtracted from that obtained from the corresponding lysates of the strain carrying p30-luc, and induction values were calculated setting the luciferase activity at OD$_{600} = 0.2$ to unity. In parallel, MicA accumulation was measured by quantitation of Northern blots and, at the same time, OD-values. Here, RNA samples were taken from the control-plasmid-carrying strain since the high-copy plasmid p30-luc might out-titrate $\sigma^{E}$, resulting in lower-than-normal MicA levels (data not shown). Figure 5A shows both the induction of luciferase activity driven by the micA promoter (values normalized to OD$_{600}$) and the MicA RNA levels from the control strain (normalized to 5S rRNA signals). Induction kinetics were similar for micA promoter activity (luc-values) and MicA accumulation. At the latest time point, MicA levels had dropped somewhat, whereas transcription activity stayed higher. Additional confirmation of direct $\sigma^{E}$ involvement emerges from the kinetics of micA transcription (assayed by luc expression from pluc-30) upon arabinose induction of plasmid pAC-rpoE4 (Figure 5B). An overnight culture of strain LMG194, harboring the two compatible plasmids, was diluted into fresh medium, split in two, and luc expression was measured $\pm \sigma^{E}$ induction at 5-min intervals. Figure 5B shows a marked increase in transcription already at 5 min post-induction compared to the non-induced strain. No significant increase in luminescence was measured in a strain carrying the promoterless plasmid pZE12b-luc (data not shown). Taken together, these results confirm that the effect of the $rpoE$ gene product on MicA levels can be accounted for, primarily or exclusively, by an increase in transcription rate.

Overexpression of $rpoE$ induces downregulation of OmpA synthesis

The antisense RNA MicA inhibits OmpA synthesis upon entry of cells into stationary phase (21,22). The results presented above suggest that increased $\sigma^{E}$ activity in stationary phase, and under certain stress conditions, is responsible for increased transcription and, thus, accumulation of MicA. Thus, increases in $\sigma^{E}$ should down-regulate de novo OmpA synthesis even in exponential phase when MicA levels are normally very low. We grew strain LMG194::pAC-rpoE4 to early exponential phase

Figure 3. Conservation of $\sigma^{E}$ recognition motifs in the micA promoter. Alignment of the micA promoter regions from several enterobacteria. Shaded boxes show regions of 100% sequence conservation. The positions of the $-35$ and $-10$ boxes, and of the transcription start site (+1), are shown. The invariable C residue in the $-10$ box is highlighted. An AT-rich element is present upstream of the $-35$ box. The consensus sequence of a $\sigma^{E}$-dependent promoter (14) is shown for comparison. Accession numbers of aligned sequences: E. coli U00096.2; Salmonella typhi AL627276.1; Serratia marcescens AJ628150.1; Shigella flexneri AE005674.1; Yersinia pestis AE017128.1.

Figure 4. Overexpression of rpoE induces accumulation of MicA. Plasmid-encoded rpoE was induced by arabinose, and samples were taken at different time points post-induction. (A) Total RNA was analyzed by Northern blot (panel A) for MicA and 5S RNA (loading controls). (-) and (+) indicate the absence or presence of arabinose. (B) The levels of $\sigma^{E}$ and $\sigma^{70}$ were determined by Western blot analysis of the corresponding total protein extracts.
in minimal medium lacking cysteine and methionine. The culture was split in two, one was induced for σE by arabinose, the other remained uninduced. Cells were pulse-labeled with 35S-Met and, subsequently, chased with a molar excess of unlabeled methionine. The labeled OmpA and σE proteins were separately immunoprecipitated from the protein extracts and analyzed on gels. A comparison of the induced and uninduced samples confirmed arabinose-dependent upregulation of σE (Figure 6, cf. lanes 1 and 2) and showed that, concomitantly, the intensity of the OmpA-specific band was decreased (cf. lanes 3 and 4). This means that, even in the exponential phase, a high activity/synthesis of σE results in higher MicA levels that in turn inhibits OmpA synthesis; this does not rule out the possibility that other σE-driven sRNAs may have minor inhibitory effects on ompA expression. Additionally, we note that induction of σE alters the protein synthesis pattern during the pulse (cf. lanes 5 and 6), in good agreement with the documented global effects expected (14,18,19).

**MicA is exclusively transcribed by σE**

The experiments above indicate that σE is responsible for MicA transcription and, hence, accumulation. σ factors are known to display redundancy; for example, the rpoS promoter is recognized by both σ5 and σ70 (29), and overlapping functions on some promoters have also been observed for σ32 and σE (28). Thus, we tested whether other σ factors could partially substitute for σE. The rpoE gene is essential in *E. coli*, but σE-deleted strains carrying an unknown suppressor mutation are available (20). Cultures of the rpoE deletion strain and its isogenic wild-type counterpart were grown, and RNA was extracted in exponential, early- and late-stationary phase. The Northern blot in Figure 7 shows induction of high MicA levels in the wild-type strain, whereas MicA was virtually absent in the rpoE strain. This suggests that other σ factors cannot substitute for micA transcription.

**DISCUSSION**

In this communication, we address the regulation of MicA RNA, the antisense regulator of the outer membrane protein OmpA. Previous work has shown that MicA accumulates before entry into stationary phase, concomitant with a decrease in the levels of the ompA mRNA and OmpA protein (21,22). The results presented here indicate that MicA accumulation is controlled at the transcriptional level, and that σE is the transcription factor responsible. Figure 1 shows that, in particular, envelope-stress-related treatments (ethanol, high salt, heat shock) caused
The OD600 values at which cultures were sampled are Northern blot, and probed for MicA (upper panel) and 5S rRNA, harvested at different stages of growth. Total RNA was analyzed by an increase in s rpoE phase. E. coli isogenic strains indicated ppGpp, the alarmone that facilitates growth-phase-dependent induction of MicA in mutant where MicA concentration is normally low. Analyses of MicA is absent in an Figure 7. MicA was virtually undetectable in a primarily or exclusively transcriptional. Finally, since MicA accumulation throughout growth, regulation must be accumulated normally in its wild-type sibling (Figure 7), regulatory proteins (35). The abundance of binding sites to regulatory complexity. A second layer of control is posttranscriptional and involves sRNAs. The ompF porin gene is controlled by EnvZ (sensor kinase)/OxyR (response regulator) and several other regulatory proteins (35). The abundance of binding sites for regulators such as HN-S, OmpR, Lrp and others, in the promoter regions of outer-membrane protein genes, testifies to regulatory complexity. A comparison of the micA promoter in E. coli to homologous regions in enterobacterial relatives showed high conservation and an excellent match to the σE consensus (Figure 3). Experimental support for σE-dependent transcription comes from induced overexpression of a plasmid-borne copy of the rpoE gene; an increase in σE level (Figure 4B) resulted in rapid accumulation of MicA (Figure 4A). Since a transcriptional micA promoter–luc reporter gene fusion showed an upregulation that was roughly proportional to MicA accumulation throughout growth, regulation must be primarily or exclusively transcriptional. Finally, since MicA was virtually undetectable in a ΔrpoE strain, but accumulated normally in its wild-type sibling (Figure 7), micA gene expression is strictly dependent on σE. Two papers published during completion of this manuscript also reported on σE-dependent micA regulation in E. coli (33) and Salmonella enterica (34). The paper by Valentin-Hansen’s group furthermore identified the RybB RNA as an additional member of the σE regulon. This sRNA targets the ompC and ompW genes (33).

Until recently, extracytoplasmic stress was believed to be solely responsible for an upregulation of σE activity. Conditions—such as heat, overproduction of OMPs, changes in osmolarity, etc.—that cause misfolded proteins to accumulate in the periplasm are sensed by the DegS protease. A proteolytic signal transduction cascade ultimately results in cleavage of the anti-σ RseA. σE is released into the cytoplasm and competes for core RNA polymerase, and this induces dramatic global changes in the cell’s expression patterns (14,19). The σE regulon comprises genes involved in maintaining or restoring membrane integrity, but also heat shock, DNA repair, chaperone and other classes of genes (14,18,19). Recently, an alternative RseA-independent pathway has been reported. Here, stationary phase growth per se may be sensed as nutritional deprivation through accumulation of ppGpp, and σE is activated by a mechanism that is not yet understood (32). With respect to MicA induction, it is likely that both pathways are used. Growth-phase-dependent upregulation of MicA is dependent on ppGpp, and induced membrane stresses likewise increase MicA concentration. In both cases, ompA regulation occurs in an hfg-dependent fashion.

It makes sense that the structure of the bacterial envelope is tightly regulated. The protein composition of the outer membrane (channels, porins, transporters) determines the flux of metabolites, salts and ions in and out of the cell. Cell-surface proteins and structures such as flagella, fimbriae and curli are also points of contact with eukaryotic cells/other bacteria/bacteriophages, and virulence can be dependent on proper outer membrane remodeling (1–3). Thus, surface properties and the appropriate levels of envelope proteins must be monitored and controlled according to the prevailing conditions. Much of this adaptive regulation is transcriptional and uses global-stress-responsive, two-component systems, σ factors and other regulatory proteins. For instance, the ompF porin gene is controlled by EnvZ (sensor kinase)/OxyR (response regulator) and several other regulatory proteins (35). The abundance of binding sites for regulators such as HN-S, OmpR, Lrp and others, in the promoter regions of outer-membrane protein genes, testifies to regulatory complexity.
strain that is incapable of mounting the \(\sigma^E\) stress response (39). This occurs by downregulation of the \(ompA\) and \(ompC\) genes, thus decreasing the abundance of misfolded OMPs. So far, RseX has not been detected from its chromosomal copy. All these sRNAs are known or suspected to act by base-pairing to target mRNAs, and appear to require Hfq for regulatory efficiency and/or intracellular stability.

Expression patterns suggest the OMP-related sRNAs to be under transcriptional control, even though the regulators responsible are in most cases unknown. MicF, OmrA and OmrB are regulated by OxyR (2). Upon oxidative stress, MicF is additionally regulated by MarR and SoxR (35). The finding that envelope stress, and entry into stationary phase, leads to a \(\sigma^E\)-dependent upregulation of MicA and RybB (this work, 33,39) is therefore not surprising.

Of the functionally assigned sRNAs in \(E.\ coli\), close to half are regulators of outer-membrane proteins. Whether this is coincidental or not is unclear. So far, many OMPs are targeted by sRNAs, and overlapping regulation is observed. For instance, both MicA and RseX can inhibit \(ompA\) gene expression (21,22,39). Conversely, one sRNA can affect the expression of multiple \(omp\) genes. The RybB RNA in \(Salmonella\ typhimurium\) downregulates more than eight \(omp\) mRNAs (3). The fitness of bacteria must be dependent on adequate transcriptional and posttranscriptional control of outer-membrane proteins. The frequent association of sRNA-genes with neighboring \(omp\) genes (which are not targeted by the sRNA) suggests a complex circuitry that can modulate membrane properties in response to changing requirements. It may well be that the \(\sigma^E\) regulon comprises additional regulatory sRNAs targeting \(omp\) mRNAs; global analyses of \(\sigma^E\)-dependent transcripts showed downregulation rather than upregulation of some \(omp\) mRNAs (14). Some of these cases may be explained by the action of hitherto unidentified \(\sigma^E\)-dependent sRNAs that would help to decrease OMP levels, thereby relieving membrane stress.

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