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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 σ^S. 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 σ^E is activated. The strict dependence of *micA* transcription on σ^E is supported by three observations. Induced overexpression of σ^E increases *micA* transcription, an Δ*rpoE* mutant displays undetectable MicA levels and the *micA* promoter has the consensus σ^E signature. Thus, MicA is part of the σ^E 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 σ^S. This transcription factor, in turn, is under

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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^S 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^{70} (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, heatshock 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 MC4100*relA*$^+$ 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 (5′-GAA CCT CGA GCT ATC TAA CAA CGG CCA TTT A; XhoI site underlined) and KU32-pMic (5′-GAT AAG AAT TCA TAT ACT CAG ACT CGC CT; 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		
MC4100*relA*1	*araD*139, Δ(*argF-lac*)205, *flb*-5301, *pstF*25, *rpsL*150, *deoC*1, *relA*1	(21)
MC4100*relA*$^+$	*relA*$^+$ derivative of MC4100	(21)
MC4100*rel*A$^+$ *rpoS*::Tn10	Sigma S deficient derivative of MC4100*relA*$^+$	Shoshy Altuvia
MC4100 *relA1spoT*	ppGpp-null derivative of MC4100	Thomas Nyström
MC4100 *relA1 cya*$^-$	cAMP – deficient MC4100	B.E. Uhlin
CAG16037	*araD* Δ(*ara-leu*)7697, Δ(*codB-lacI*), *galK*16, *galE*15, *mcrA*0 *relA*1, *rpsL*150, *spoT*1, *mcrB*9999, *hsdR*2 [*φλrpoH* P3::*lacZ*]	(40)
CAG22216	CAG16037 Δ*rpoE*	(41)
LMG194	F– Δ*lacX*74, *galE*, *thi, rpsL*, Δ*phoA*, Δ*ara*714, *leu*::*Tn*10	Invitrogen
GW 986	LMG194 (pACYC184)	This work
GW 969	LMG194 (pAC-rpoE4)	This work
GW 962	MC4100*relA*$^+$ (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 L 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 MC4100*relA*$^+$ 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 (hyperosmolarity). 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. One-third of the culture each was transferred to fresh Erlenmeyer flasks pre-incubated in waterbaths at 30°C, 37°C or 42°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 $OD_{600} \sim 0.2$, 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 vol 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 vol 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-^{32}P-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% (v/v) Tween20). Blots were incubated in BLOTTO with rabbit monoclonal antibodies targeting σ^E or σ^{70} (Neoclone). Dilutions of antibody were 1:1000 (σ^E) or 1:10000 (σ^{70}). 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 1170-1-0000 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 $OD_{600} \sim 0.3$, 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 MC4100*relA*$^+$ 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*$^+$/Δ*rpoS*, 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 Δ*rpoS* than in an *rpoS*$^+$ background.

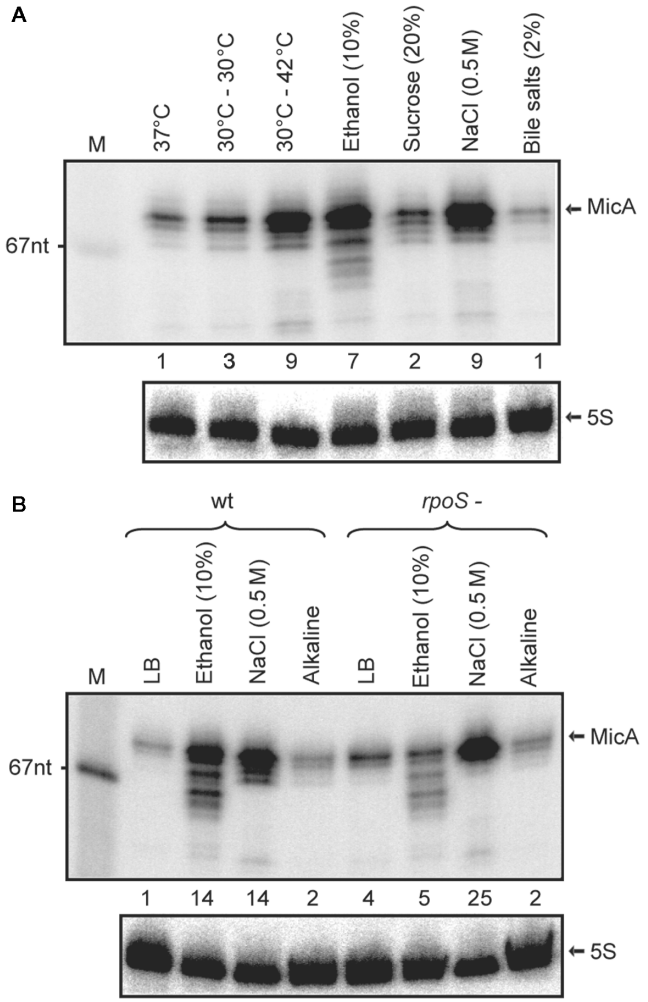

Figure 1. MicA levels under various stress conditions. Northern-blot analysis was carried out on total RNA extracted from exponential phase *E. coli* cells exposed to various stress conditions. Membranes were hybridized with probes against MicA (upper panels) or 5S rRNA as loading controls (lower panels). (**A**) Different stress treatments of strain MC4100*relA*$^+$ as indicated; (**B**) Stress treatments in either *rpoS*-proficient or -deficient strains. Treatment protocols are detailed in Materials and Methods. Induction levels of MicA (ratios: treated/control) are indicated between panels.

E. coli mutant strains exhibit aberrant MicA levels

Earlier attempts to transcribe the *micA* gene *in vitro* by RNA polymerase holoenzyme (containing σ^{70}) 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^{70} for alternative σ factors, is thought to be dependent on the nutritional stress signal ppGpp (27). Thus, we monitored the growth-phase-dependent

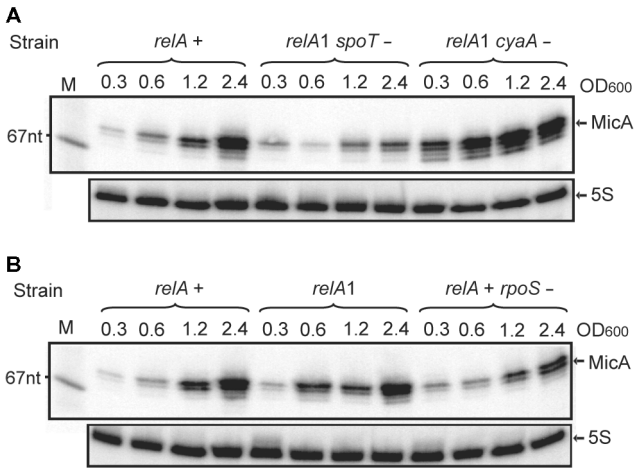

Figure 2. Induction of MicA accumulation in wild-type and mutant *E. coli* strains. Cultures of bacteria were grown and aliquots were withdrawn at the indicated OD_{600} values. Ten micrograms of RNA from each sample was run on denaturing 6% polyacrylamide gels, transferred to charged nylon membranes and probed for MicA RNA (upper panel) or 5S rRNA as loading controls (lower panel).

accumulation of MicA in strains carrying mutations that decrease or abolish ppGpp synthesis (*spoT*, *relA*1), 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 *relA*1 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 *relA*1 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 σ^{70} promoters are known to be affected by this alarmone as well. Compared to its wild-type counterpart, the *rpoS* mutant strain showed, however, only a modest reduction of ~40% (Figure 2B, right panel). Thus, σ^S 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, OD_{600} 0.3) compared to wild-type (lane 2). MicA levels in the *cyaA* mutant remained high throughout growth until $OD_{600} \sim 2.4$. At this point in the growth curve, the MicA level of the wild-type strain approached that of the *cyaA* strain. The possible involvement of catabolite repression, which is relieved in a *cyaA* 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 σ^E 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 σ^E 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 σ^E 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 σ^E. 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 σ^E recognition (30). Spacer and discriminator regions are of median size at 16 and 6 bp, respectively. The −10 box contains an invariable C-residue found in σ^E-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 σ^E.

Overexpression of σ^E results in increased MicA accumulation

In order to ascertain σ^E-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 ~fivefold higher than in the uninduced samples. Figure 4B shows a Western blot analysis of total protein lysate from the same time points confirming

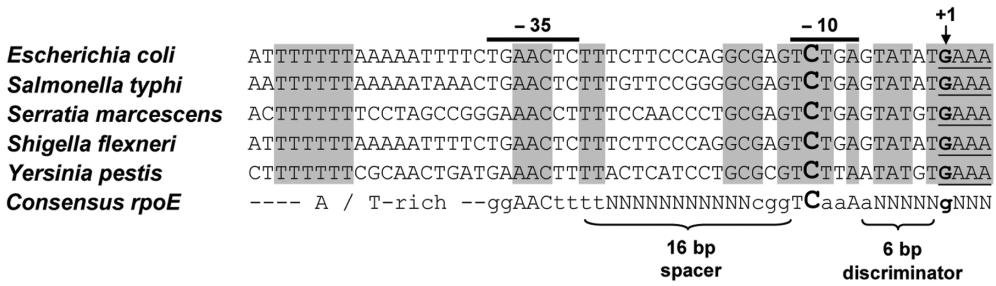

Figure 3. Conservation of σ^E recognition motifs in the *micA* promoter. Alignment of the *micA* promoter regions from several enterobacteria. Shaded boxes show regions of 100% sequence conservation. The positions of the −35 and −10 boxes, and of the transcription start site (+1), are shown. The invariable C residue in the −10 box is highlighted. An AT-rich element is present upstream of the −35 box. The consensus sequence of a σ^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.

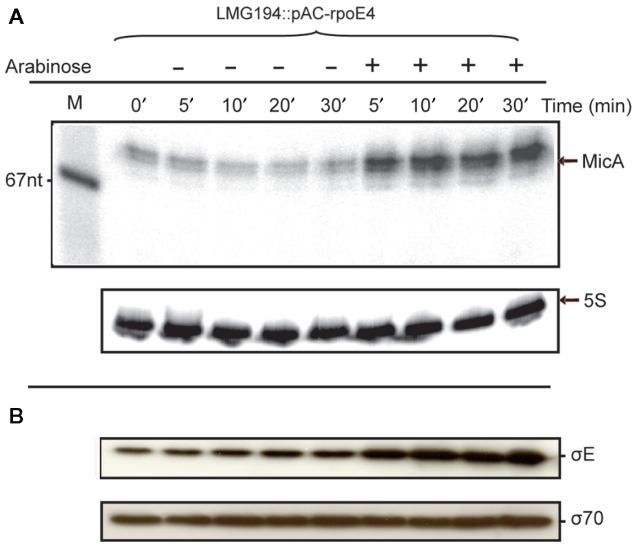

Figure 4. Overexpression of rpoE induces accumulation of MicA. Plasmid-encoded rpoE was induced by arabinose, and samples were taken at different time points post-induction. (**A**) Total RNA was analyzed by Northern blot (panel A) for MicA and 5S RNA (loading controls). (−) and (+) indicate the absence or presence of arabinose. (**B**) The levels of σ^E and σ^{70} were determined by Western blot analysis of the corresponding total protein extracts.

the induction of σ^E, whereas the level of the housekeeping σ^{70} was essentially unaffected.

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

The experiment in Figure 4 suggests that σ^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 σ^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 σ^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 σ^E activity in stationary phase, and under certain stress conditions, is responsible for increased transcription and, thus, accumulation of MicA. Thus, increases in σ^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

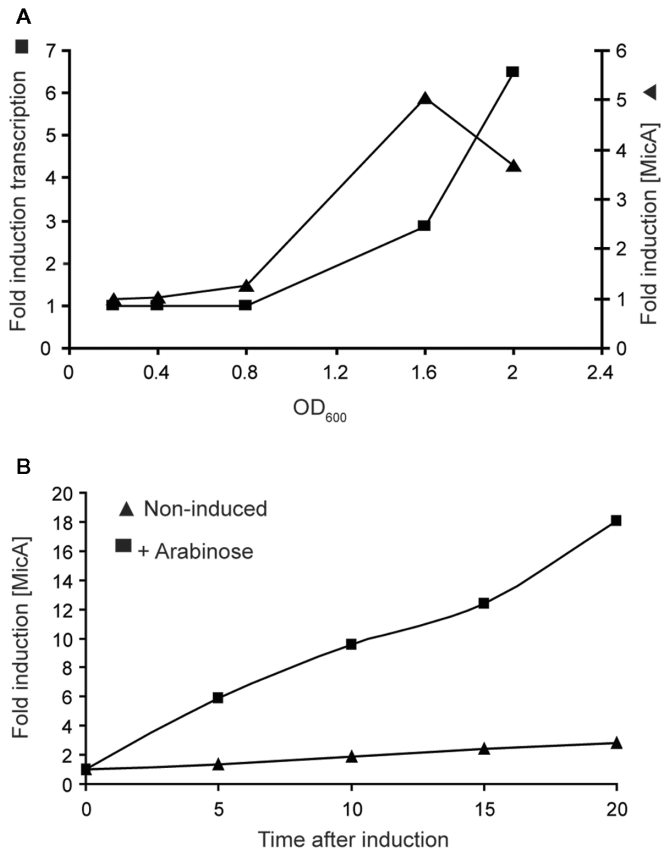

Figure 5. MicA accumulation and transcription rate in different growth phases. (**A**) Exponentially growing *E. coli* cells either carrying the *micA* promoter-*luc* fusion plasmid pluc-30, or the promoterless control plasmid, were assayed for specific luciferase activity at the OD_{600} values indicated (squares). Values depicted show values relative to that obtained at $OD_{600} = 0.2$. In parallel, MicA accumulation was quantified by Northern analysis, and normalized to 5S rRNA signals. These values are likewise shown as relative induction, compared to that at $OD_{600} = 0.2$ (triangles). (**B**) The kinetics of σ^E-dependent induction of MicA transcription was analyzed using strain LMG194::pAC-rpoE4, carrying in addition the reporter plasmid pluc-30. At time 0, the culture was either induced (squares) or mock-treated (triangles). Samples were taken for determination of luciferase activity at the indicated time points. Values show relative increases over that obtained at $t = 0$ min.

in minimal medium lacking cysteine and methionine. The culture was split in two, one was induced for σ^E by arabinose, the other remained uninduced. Cells were pulse-labeled with ^{35}S-Met and, subsequently, chased with a molar excess of unlabeled methionine. The labeled OmpA and σ^E 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 σ^E (Figure 6, cf. lanes 1 and 2) and showed that, concomintantly, 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 σ^E results in higher MicA levels that in turn inhibits OmpA synthesis; this does not rule out the possibility that other σ^E-driven sRNAs may have minor inhibitory effects on *ompA* expression. Additionally, we note that induction

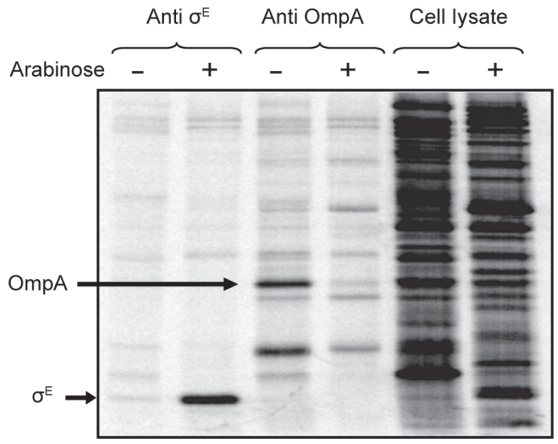

Figure 6. Downregulation of OmpA synthesis by rpoE induction. A pulse-chase experiment was conducted with or without *rpoE* overexpression, as described in Materials and Methods. The figure shows a gel analysis of ^{35}S-Met-labeled protein immunoprecitated by antibodies against either σ^E or OmpA. The bands corresponding to these proteins on the gel are indicated. The OmpA antibody cross-reacts with some other proteins. The two rightmost lanes show the total protein patterns under inducing and control conditions prior to immunoprecipitation.

of σ^E 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 σ^E

The experiments above indicate that σ^E is responsible for MicA transcription and, hence, accumulation. σ factors are known to display redundancy; for example, the *rpoS* promoter is recognized by both σ^S and σ^{70} (29), and overlapping functions on some promoters have also been observed for σ^H and σ^E (28). Thus, we tested whether other σ factors could partially substitute for σ^E. The *rpoE* gene is essential in *E. coli*, but σ^E-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 Δ*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 σ^E is the transcription factor responsible. Figure 1 shows that, in particular, envelope-stress-related treatments (ethanol, high salt, heat shock) caused

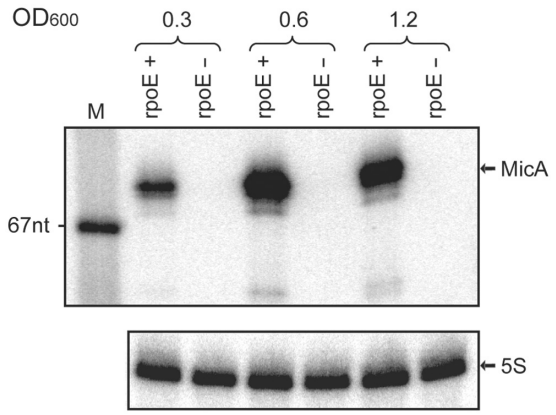

Figure 7. MicA is absent in an *rpoE* mutant strain. Cultures of an isogenic *E. coli* strain pair, $rpoE^{+}/rpoE^{-}$, were grown in LB and harvested at different stages of growth. Total RNA was analyzed by Northern blot, and probed for MicA (upper panel) and 5S rRNA, (lower panel). The OD_{600} values at which cultures were sampled are indicated and represent exponential, early stationary and stationary phase.

upregulation of MicA in exponential phase—a condition where MicA concentration is normally low. Analyses of growth-phase-dependent induction of MicA in mutant strains indicated ppGpp, the alarmone that facilitates σ factor exchange on RNA polymerase, to be required for upregulation. In contrast, the presence or absence of the stationary phase/stress σ^S had only small effects on induction (Figure 2). The observation that a Δ*cyaA* strain displayed high levels of MicA even in exponential phase (Figure 2) was initially puzzling. Recently, however, it was found that the absence of cAMP results in strong induction of σ^E activity (31); the mechanism by which this occurs is as yet unknown. Since the MicA-inducing stresses (in Figure 1) as well as stationary-phase growth have previously been associated with an increase in synthesis and/or activity of the ECF σ^E (16,32), this factor is strongly suggested to be involved in *micA* regulation.

The recent characterization of σ^E-driven promoters identified characteristic recognition motifs (14,19). A comparison of the *micA* promoter in *E. coli* to homologous regions in enterobacterial relatives showed high conservation and an excellent match to the σ^E consensus (Figure 3). Experimental support for σ^E-dependent transcription comes from induced overexpression of a plasmid-borne copy of the *rpoE* gene; an increase in σ^E 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 Δ*rpoE* strain, but accumulated normally in its wild-type sibling (Figure 7), *micA* gene expression is strictly dependent on σ^E. Two papers published during completion of this manuscript also reported on σ^E-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 σ^E regulon. This sRNA targets the *ompC* and *ompW* genes (33).

Until recently, extracytoplasmic stress was believed to be solely responsible for an upregulation of σ^E 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. σ^E 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 σ^E 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 σ^E 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 *hfq*-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.

A second layer of control is posttranscriptional and involves sRNAs. The *ompF* gene was found to be regulated by the sRNA MicF, the founding member of the chromosomally encoded antisense RNAs (36). Work in recent years has extended the list of OMP-regulatory sRNAs. MicC and MicA are antisense inhibitors of *ompC* and *ompA* porin gene expression (21,22,37). OmrA and OmrB, two sRNAs of almost identical sequence but with different expression patterns, downregulate the mRNA levels of at least four OMP genes, *ompT*, *fecA*, *cirA* and *fepA* (38), possibly by an antisense mechanism. RybB downregulates *ompC* and *ompW* in *E. coli* (33), and more than eight genes in *Salmonella* (3). The RseX RNA, when ectopically expressed at high copy, rescues an *rseP* mutant

strain that is incapable of mounting the σ^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 σ^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 σ^E regulon comprises additional regulatory sRNAs targeting *omp* mRNAs; global analyses of σ^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 σ^E-dependent sRNAs that would help to decrease OMP levels, thereby relieving membrane stress.

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