Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon

Emily B. Gogol[a,b,c], Virgil A. Rhodius[c], Kai Papenfort[d], Jörg Vogel[d,1], and Carol A. Gross[c,e,1]

[a]Biomedical Sciences Graduate Program, [b]Program in Microbial Pathogenesis and Host Defense, [c]Department of Microbiology and Immunology, and [e]Department of Cell and Tissue Biology, University of California, San Francisco, CA 94158; and [d]Institute of Molecular Infection Biology, University of Würzburg, Würzburg D-97080, Germany

Contributed by Carol A. Gross, June 20, 2011 (sent for review May 10, 2011)

The *Escherichia coli* σ^E envelope stress response monitors and repairs the outer membrane, a function central to the life of Gram-negative bacteria. The σ^E stress response was characterized as a single-tier activation network comprised of ~100 genes, including the MicA and RybB noncoding sRNAs. These highly expressed sRNAs were thought to carry out the specialized function of halting de novo synthesis of several abundant porins when envelope homeostasis was perturbed. Using a systematic target profiling and validation approach we discovered that MicA and RybB are each global mRNA repressors of both distinct and shared targets, and that the two sRNAs constitute a posttranscriptional repression arm whose regulatory scope rivals that of the protein-based σ^E activation arm. Intriguingly, porin mRNAs constitute only ~1/3 of all targets and new nonporin targets predict roles for MicA and RybB in crosstalk with other regulatory responses. This work also provides an example of evolutionarily unrelated sRNAs that are coinduced and bind the same targets, but at different sites. Our finding that expression of either MicA or RybB sRNA protects the cell from the loss of viability experienced when σ^E activity is inadequate illustrates the importance of the posttranscriptional repression arm of the response. σ^E is a paradigm of a single-tier stress response with a clear division of labor in which highly expressed noncoding RNAs (MicA, RybB) endow a transcriptional factor intrinsically restricted to gene activation (σ^E) with the opposite repressor function.

seed pairing | sigma factor

Bacteria respond to cellular stresses and environmental cues by altering the activity of transcription factors. The mode of DNA binding by the transcription factor determines whether it is an activator, repressor or both. Dual activity can be advantageous because it permits simultaneous activation of some genes while repressing incompatible genes and boosts regulatory versatility within a transcriptional network by increasing the achievable number of network motifs in bacteria (e.g., feed-forward loops; ref. 1). The alternative solution, recruiting an opposite regulatory activity through a downstream transcription factor, is rare in bacteria.

The σ^E response to envelope stress is one of the best characterized bacterial transcription programs (2). σ^E is sequestered in an inactive form at the inner membrane under nonstress conditions. Perturbation of envelope homeostasis, caused by damage of the outer membrane (OM) or the accumulation of unfolded outer membrane proteins (OMPs) such as porins, triggers release of σ^E to the cytoplasm, where it directs RNA polymerase to transcribe the σ^E regulon (Fig. 1). Promoters recognized by σ^E have been mapped to saturation in *Escherichia coli*, revealing that σ^E directly activates ~60 transcriptional units that comprise a total of ~100 genes (3, 4). The few targets with transcriptional function (*rpoE*, *rpoH*, *greA*) potentiate positive regulation, suggesting that the σ^E network is restricted to transcriptional activation. Thus, repressors boosting complexity must operate posttranscriptionally. Intriguingly, the distribution of promoter strengths in the σ^E regulon suggests candidates for such repressors (Fig. 1), as two of the three strong promoters transcribe small noncoding RNAs (sRNAs).

These two sRNAs, MicA and RybB, are conserved in many enterobacteria and belong to the growing class of sRNAs associated with the RNA chaperone Hfq that use short base pairing interactions to modulate the translation and decay rates of *trans*-encoded target mRNAs (5, 6). Previous studies of MicA and RybB in *E. coli* and *Salmonella* showed that both repress the synthesis of several major OMPs by binding in the 5′ mRNA region (7–15). This has led to a simplistic model that the specialized function of these sRNAs is to halt de novo synthesis of very abundant OMPs upon σ^E induction. However, the full target suites of MicA and RybB were unknown, and biocomputational algorithms readily predicted many additional mRNA interactions. Additionally, Hfq and σ^E oppositely regulated a number of *E. coli* mRNAs, as expected if they were MicA and RybB targets (16). Finally, σ^E induced repression of several *E. coli* mRNAs, such as *ompX* and *fiu*, required Hfq, again suggesting regulation by a σ^E-dependent sRNA (4, 16).

Using a systematic target profiling and validation approach, we discovered that MicA and RybB are each global repressors of both distinct and shared targets, the latter resulting in convergent target regulation by bacterial sRNAs. These two noncoding regulators constitute a posttranscriptional repression arm that is of roughly comparable regulatory scope to the protein-based transcriptional activation arm of the σ^E response, playing a far broader role than simply preventing the accumulation of unassembled OMPs. We demonstrate that it is the combined activity of the activation and repression arms that enables single-tier transcription factor σ^E to monitor and maintain a trait as complex as envelope homeostasis.

Results

Quality Control Functions of MicA and RybB Are Central to the σ^E Response. Lack of σ^E is lethal to *E. coli* (17). Analyses of suppressors of *rpoE* deletion strains suggested that lethality results from induction of a cell death pathway as a result of imbalanced expression of other envelope stress responses (18, 19). To evaluate the physiological impact of MicA and RybB, we used cell death as readout after targeted shutoff of σ^E following overexpression of its two antagonists, RseA and RseB. When active σ^E is depleted by RseA/B overexpression, growth ceases prematurely and viability

Author contributions: E.B.G., J.V., and C.A.G. designed research; E.B.G. and K.P. performed research; E.B.G. contributed new reagents/analytic tools; E.B.G., V.A.R., and K.P. analyzed data; and E.B.G., J.V., K.P., and C.A.G. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed: E-mail: joerg.vogel@uni-wuerzburg.de or cgrossucsf@gmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109379108/-/DCSupplemental.

MICROBIOLOGY

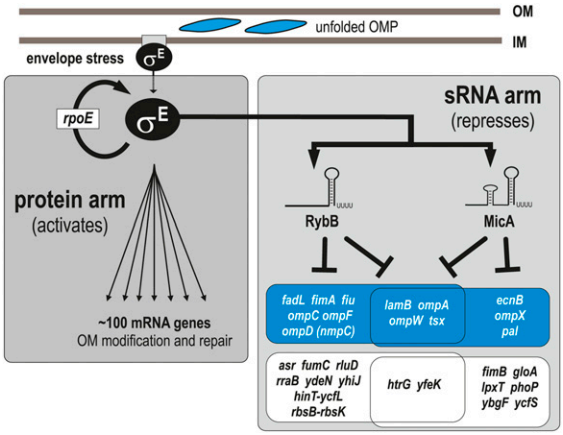

Fig. 1. Initiation of the σ^E response and its immediate effects. This figure illustrates how the σ^E response has both activator (protein arm) and repressor (sRNA arm) functions that act primarily to survey and maintain cell envelope homeostasis. Genes targeted for down-regulation by the known σ^E-dependent sRNAs are shown in the Venn diagrams; those in blue are outer membrane proteins or lipoproteins associated with the cell envelope.

decreases (Fig. 2), as reported in ref. 19. Importantly, concomitant overexpression of either MicA or RybB rescues both growth and viability phenotypes exhibited following σ^E shutoff (Fig. 2 *A* and *B*). Rescue by MicA or RybB does not result from inadequate inactivation of σ^E: Upon RseAB overexpression, σ^E activity is similarly low whether or not MicA or RybB is overexpressed (Fig. 2*C*). Together, these results indicate that the repressor function of each sRNA provides σ^E with an immediate stress reduction response to imbalances in the OM that is sufficient to avert cell death. Parenthetically, as expected, overexpression of either MicA or RybB prevents the normal growth-phase dependent increase in σ^E activity (Fig. 2*C*), most likely because reducing OMP synthesis is known to decrease σ^E activity (2).

Combinatorial Target Searches Identify MicA and RybB as Regulators With Global Reach. To comprehensively define the target suite of the two sRNAs, we used high-density tiling arrays to identify changes in mRNA abundance after short overexpression of MicA or RybB from inducible plasmids. We used four different conditions to accommodate the possibility that regulation was growth-phase specific (exponential vs. stationary phase), as noted previously for the Hfq-associated ArcZ sRNA (20), or media specific (glucose vs. maltose; columns 1–6 of Table S1, Table S2, and Fig. S1*A*).

We identified 31 regulated mRNAs, all of which were negatively regulated; 80% responded in at least three conditions, and ~20% were condition specific. Quantitative real-time PCR (qRT-PCR) validated that RybB regulated 16 genes; MicA regulated 9 genes; and both sRNAs regulated 6 genes (Fig. 1). Importantly, although previous results suggested that RybB had only two targets in *E. coli* (10), the candidate targets in the new data set include all *Salmonella* targets identified for RybB (7, 11). An exception is the *ompN* mRNA, which was not regulated in *E. coli*, likely because of two critical bases in the mapped RybB site that differ from *Salmonella ompN* (Fig. S1C; ref. 9). As suggested previously (16), the *yhcN* and *lpp* mRNAs gave a very small (≤twofold) change to both σ^E or sRNA overexpression, suggesting little regulation, or regulation taking place solely at the level of translation (Fig. S1C). MicA and RybB not only down-regulate all major *E. coli* porins, but also have many additional candidate targets, including some without envelope related functions. Thus, the two sRNAs are global regulators, controlling many mRNAs transcribed from physically unlinked genes.

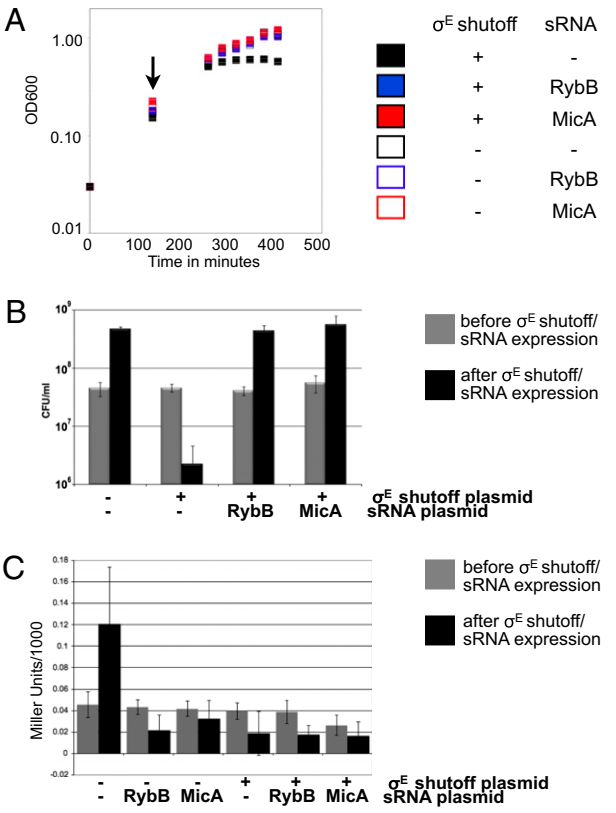

Fig. 2. RybB and MicA protect cells from lysis during σ^E shutoff. The growth (*A*), viability (*B*), and σ^E activity (*C*) of strains before and after overexpression of RseA/B in the σ^E shutoff plasmid (+: contains σ^E shutoff plasmid; –: empty vector plasmid) without (–sRNA plasmid) or with concomitant overexpression of the plasmid-encoded sRNA (RybB or MicA). *rseAB* as well as MicA and RybB are controlled by IPTG inducible promoters. (*A*) The strain with the σ^E shutoff plasmid only (+σ^E shutoff, –sRNA plasmid; filled black squares) exhibited decreased growth upon σ^E shutoff; all other strains grew almost identically as shown by the overlapping symbols. (*B*) The strain with σ^E shutoff plasmid only (+σ^E shutoff, –sRNA plasmid) showed reduced colony forming units following σ^E shutoff. Concomitant overexpression of either sRNA (+σ^E shutoff, RybB; +σ^E shutoff, MicA) fully restored viability. (*C*) σ^E activity of each strain shown in *A* both before and after σ^E shutoff/sRNA overexpression was determined from the β-galactosidase activity of a chromosomally encoded σ^E dependent *rpoHP3-lacZ* reporter. Bacteria grown overnight at 30 °C in LB with ampicillin and chloramphenicol were subcultured to OD_{600} = 0.03 in fresh media and grown at 30 °C. IPTG (1 mM) was added just before 135 min of growth (OD_{600} ~ 0.1) to induce overexpression of RseA/B, MicA and RybB, as indicated by the arrow. The "–" sample was taken just before 135 min of growth, and the "+" sample was taken at 255 min of growth. The average of three experiments with SD is shown.

We determined whether targets were also repressed by overexpression of σ^E, as expected because this condition induces chromosomal MicA and RybB (10, 12, 21). Many targets (15 in total) were significantly repressed under this condition (column 2 of Table S1), but three targets were up-regulated (*htrG*, *yfeK*, and *yhjJ*). All three genes have upstream σ^E-dependent promoters (4), leading to net up-regulation irrespective of concomitant post-transcriptional repression of their mRNAs by MicA and/or RybB. No significant σ^E-dependent regulation was observed for 13 targets. Our additional experiments validate these as direct targets (see below). The most likely explanation is that MicA or RybB primarily act to repress translation of these targets, so that decreased mRNA levels are apparent only when the sRNAs are highly overexpressed. This explanation is in line with previous studies indicating that, when regulation is primarily translational, changes in mRNA levels are below the threshold for significance

in microarray studies (22, 23). Alternatively, compensatory activities in the regulon may mask repression. A time course of σ^E overexpression for select targets indicated some temporal distinction in the dynamics of mRNA level changes (Fig. S2). For example, *nmpC* was fourfold repressed after 5 min of σ^E overexpression, which is 50% of the repression level observed after 20 min of σ^E overexpression. In contrast, *ompF* exhibited little repression at 5 min, but was repressed 64-fold after 20 min of σ^E overexpression.

Conserved 5′ End of RybB Regulates Many New Targets in *E. coli*. Analysis of *Salmonella* RybB established that the highly conserved nucleotides 1–16 of RybB (called R16) is usually sufficient for target repression, and that regulation critically depends on the GCC motif at the very 5′ end of RybB (7, 11). The *RNAhybrid* algorithm (24) predicts that most newly discovered RybB targets are also guided by R16 (Fig. S3). Therefore, we tested the importance of R16 and the GCC motif by comparing target repression by authentic RybB with two variants, RybB-M2 and R16TOM (Fig. 3). RybB-M2 has a C_2-to-G change, thereby disrupting its 5′ terminal GCC motif. R16TOM is a fusion of R16 to TOM, an unrelated control sRNA derived from 5′ truncation of *E. coli* OmrB sRNA (9, 25, 26). Of the 17 candidate targets predicted to use the R16 region of RybB, 16 of 17 are significantly down-regulated by R16TOM (exception *ydeN*; Fig. 3*A*). Moreover, RybB-M2 (C_2-to-G change) is unable to down-regulate 13 of 14 targets predicted to use RybB C_2 for interaction (exception *ompW*), whereas 3 of 4 targets predicted not to use mutation RybB C_2 (*lamB*, *fimA*, *ydeN*) maintain down-regulation (Fig. 3*A*). These results strongly argue that R16 and the GCC motif are critical for repression (columns 8–13 of Table S1).

To prove direct target regulation in vivo, we used a well established reporter assay where a sRNA is coexpressed with a translational fusion of the target 5′ mRNA region to green fluorescent protein (GFP; ref. 27). This assay validated *E. coli nmpC*, *ompC*, and *ompF* as direct RybB targets (Fig. S4). Note that our data revise a previously proposed RybB–*ompC* pairing (10) showing that RybB recognized *ompC* in the upstream 5′ UTR, as it does in *Salmonella* (7, 11). We also validated two new targets, *fiu* and *rluD* (Fig. 3 *B* and *C*), encoding a catecholate siderophore receptor in the OM and a conserved cytosolic 23S rRNA pseudouridine synthase, respectively. Mutant RybB-M2 failed to repress the *fiu::gfp* or *rluD::gfp* fusions. Importantly, compensatory M2′ alleles of the two target fusions, predicted to restore base pairing (G-to-C change at positions −71 (Fig. 3*B*) or +4 (Fig. 3*C*) relative to the AUG of *fiu* or *rluD*, respectively) restored target repression but were now insensitive to wild-type sRNA. These results validate the predicted short RNA duplexes of R16 with these targets. Taken together, the weight of our experimental data suggests that almost all RybB regulated mRNAs (Table S1) are direct targets.

MicA Is a Global Regulator. Our microarray analysis predicted 15 candidate targets for *E. coli* MicA, significantly more than previously known in any organism (Fig. 1). Using conservation of MicA sequences as a guide (Fig. 4*A*), we constructed a series of MicA truncations/mutations (schematized in Fig. 4*A*), and examined their repression capacity to identify critical features of MicA. All 10 targets tested were down-regulated by the highly conserved nucleotides 1–24 and half were also down-regulated by nucleotides 8–24 (Fig. 4*B*; Table S1, columns 8–10; note that *ycfS* was regulated by the TOM scaffold RNA alone, and therefore could not be assessed by this procedure, Fig. S5). We expected that MicA nucleotides 1–7 would be dispensable for some targets based on validated biochemically mapped interactions (i.e., nucleotides 1–7 do not interact with *ompA* and *lamB*) and our computationally predicted pairings (e.g., no predicted interaction of MicA nucleotides 1–6 with *htrG*). However, the sufficiency of MicA 8–24 was surprising for other targets. Both *phoP* and *yfeK* are examples of targets that are predicted to interact with nucleotides 3–7 of MicA, and *phoP* interaction with nucleotides 4–7 of MicA has been validated (28).

Our mutational analysis of the GC cluster, CGCGC, spanning nucleotides 7–11, may explain this discrepancy. Given our deletion data for MicA, predicted pairings between MicA and its targets, and the fact that a GC cluster had been observed to be crucial in RybB–target interactions, we addressed the importance of MicA C_7 and C_{11} in target recognition. Down-regulation was abrogated by mutational change when pairing was predicted (6/6 for C_7-to-G; 10/10 for C_{11}-to-G; Fig. 4*C*), and maintained for all 4 targets predicted not to use C_7 (Fig. 4*C*, Table S1, columns 11–13). These mutational results provide evidence that the 5′ proximal GC cluster of MicA is an important determinant for target recognition. These results also indicate that whereas loss of pairing of C_7 is tolerated (Fig. 4*B*), an unpaired G nucleotide in both MicA and the target is not. The G–G clash at position 7 may have a negative impact on the ability of the remainder of the MicA GC cluster to pair with target, thereby

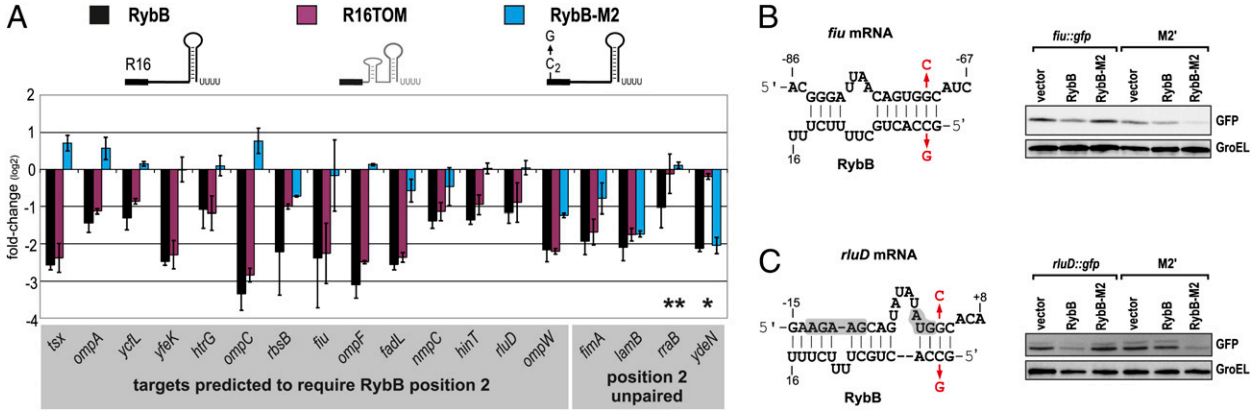

Fig. 3. Region and nucleotide specific binding by RybB. Regulation of select mRNAs targeted by RybB after overexpression of RybB, R16TOM or RybB-M2 as indicated in *A*. Data shown are the average of three experiments with SD. The double asterisk indicates R16TOM should not be sufficient to regulate *rraB*, and the single asterisk indicates R16TOM should be sufficient to regulate *ydeN*. A schematic of interaction map and the mutations used for validation are depicted for *fiu* (*B Left*) and *rluD* (*C Left*). Experimental results from *gfp* translational reporters monitored by Western blot for *fiu* (*B Right*) and *rluC* (*C Right*). Bacterial growth, induction, qRT-PCR, and analysis are described in *SI Materials and Methods*.

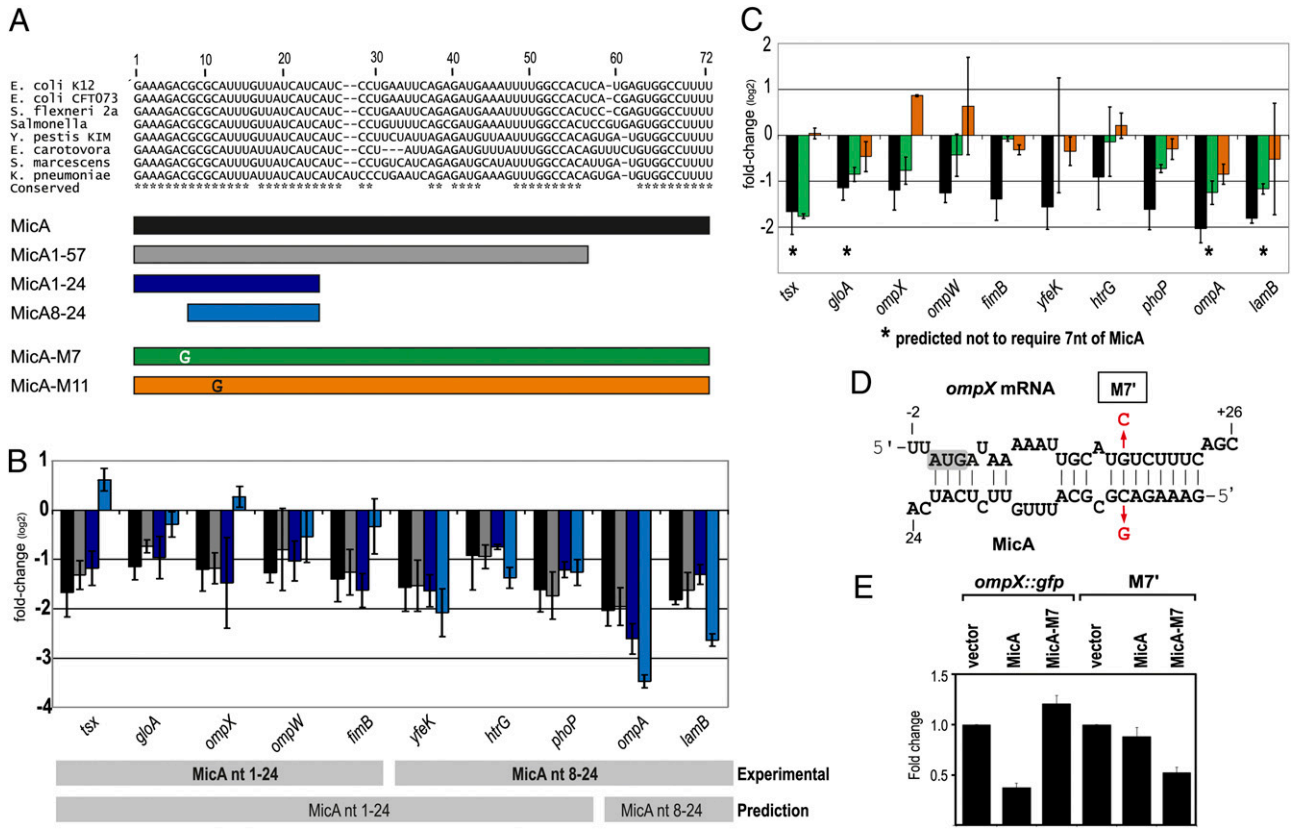

Fig. 4. Region and nucleotide specific binding by MicA. (*A*) The conservation of MicA sequence and schematized deletions and mutations to study MicA function. (*B*) Regulation of select mRNAs targeted by MicA after overexpression of full-length and truncated MicA constructs. (*C*) The effect of point mutations at MicA position 7 and 11; see *SI Materials and Methods* for details. (*D*) The proposed interaction map of MicA and OmpX and the mutations used for validation. (*E*) Fluorescence readings from *gfp* translational reporters. Data are expressed as the fold change relative to a strain expressing the *gfp* reporter only. All data shown are the average of three experiments with SD.

abrogating repression. In contrast, binding at adjacent positions of the GC cluster may still allow repression even though the initial base-pairing interaction was eliminated.

We experimentally validated one new target predicted to depend on both C_7 and the very 5′ end of MicA (*ompX*; Fig. 4*D*). Using an *ompX::gfp* fusion and a compensatory M7′ allele, we demonstrated that C_7 is essential for the repression of this target (Fig. 4*E*), which gives us a clue as to why the 5′ terminal positions of MicA are highly conserved (Fig. 4*A*).

Convergent Target Regulation by MicA and RybB. Our results suggest that MicA and RybB regulate some targets in common (Fig. 1). We tested *tsx* and *ompA* for joint regulation by both sRNAs (Fig. 5). For *ompA*, we examined the predicted interaction with RybB (Fig. 5*A*),

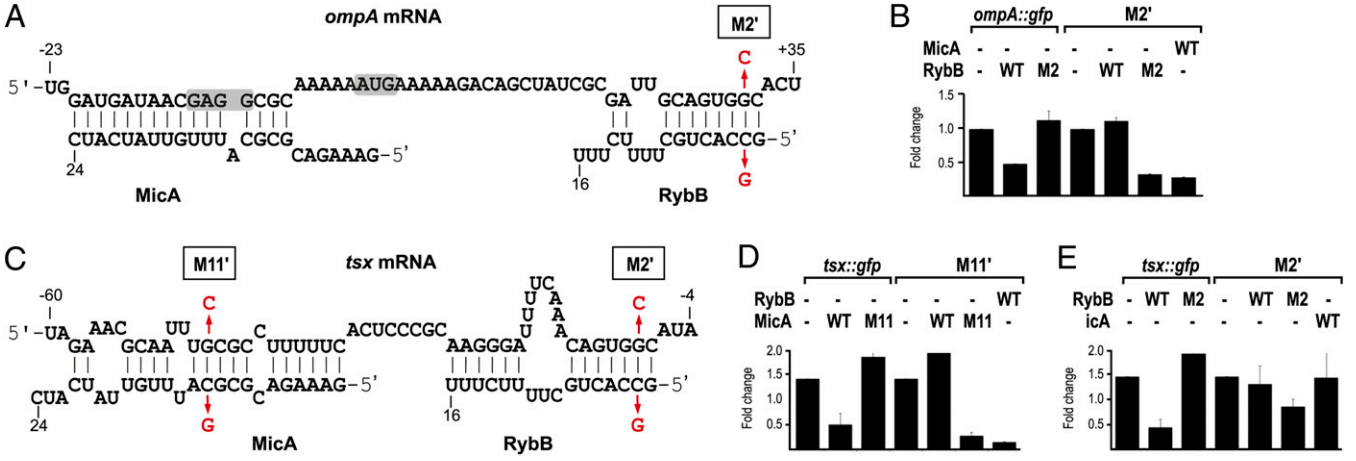

Fig. 5. Convergent target regulation by MicA and RybB Dual regulation of *ompA* and *tsx* by MicA and RybB. Schematics of proposed RNA/target interactions and the mutation changes used for validation are shown for *ompA* (*A*) and *tsx* (*C*). Results from *gfp* transational reporter assays using these constructs performed as described in Fig. 4*E* are shown in for RybB/*ompA* (*B*), MicA/*tsx* (*D*), and RybB/*tsx* (*E*).

as the MicA–*ompA* duplex is already well defined (13, 14). RybB regulation of *ompA* was disrupted by a M2 or M2′ mutation in sRNA or target, respectively, yet restored upon combining both mutations (RybB-M2, *ompA*-M2′::*gfp*; Fig. 5*B*). Significantly, a mutation in the RybB site of *ompA* mRNA has no effect on its regulation by MicA (MicA, *ompA*-M2′::*gfp*). In other words, *ompA* is subject to both parallel yet independent regulation by MicA and RybB.

We used the same strategy to validate the MicA binding site on *tsx* (Fig. 5*C*). Notably, a mutation in the MicA binding site has no effect on the ability of RybB to interact with *tsx* mRNA (Fig. 5*D*: RybB, *tsx*::*gfp* M11′), which argues that the predicted site for RybB is clearly distinct from that of MicA, and that *tsx* also is subject to dual sRNA regulation. The RybB-*tsx* pairing (Fig. 5*C*) has been validated in *Salmonella* using RybB-M2 and a compensatory *tsx*-M2′ allele (11). Surprisingly, although the nucleotides involved are conserved both between *E. coli* and *Salmonella*, we found that a M2′ allele of *E. coli tsx* was regulated by neither RybB-M2 nor MicA (Fig. 5*E*). The latter indicates that *tsx* M2′ may have an altered mRNA structure and that other strategies will have to be used to study dual sRNA control of this target in *E. coli*.

Discussion

The σ^E envelope stress response, central to the life of Gram-negative bacteria, had been seen as a single-tier regulatory network, with a positive regulator (σ^E) sufficient to monitor and repair the OM. The possibility of a more complex architecture was indicated by the fact that σ^E also activated two sRNAs, MicA and RybB, whose promoters are the strongest in the σ^E regulon (3, 4, 29). Here, we show that MicA and RybB are global regulators that together target >30 mRNAs of *E. coli*. This post-transcriptional noncoding RNA repression arm is of roughly comparable regulatory scope to the protein-based transcriptional activation arm, which consists of ~100 genes. Moreover, the two arms of the response have distinct functions. The protein activation arm controls core elements of the envelope assembly machinery (30, 31), whereas the repression arm alleviates stress and interconnects regulatory networks (see below). The physiological importance of the sRNA arm is graphically illustrated by our demonstration that expression of either MicA or RybB sRNA protects the cell from the loss of viability experienced when σ^E activity is inadequate. Thus, the σ^E stress response is a paradigm for how a noncoding RNA component endows a transcriptional activation pathway with an essential repressor function (32). Interestingly, the unfolded protein response (UPR) that counteracts protein folding stress in the endoplasmic reticulum (ER) compartment of metazoan cells also involves a dual response strategy: Transcription factors up-regulate protein folding chaperones and catalysts; simultaneously, protein synthesis is down-regulated by a separate pathway to stem the flow of precursors into the ER (33).

This study clearly establishes that MicA and RybB are global regulators of the σ^E response, both with a large suite of targets. We confirm and expand the notion that repression of porins is a major function of these sRNAs. They regulate every major porin, including OmpX, the archetypal porin stimulus of σ^E activity (34, 35). However, porins are only 30% of the sRNA targets, indicating that the scope of the sRNA response extends considerably beyond porin control. The sRNAs regulate several genes previously found to be involved in increased production of outer membrane vesicles (porins, *ycsF*, *pal*, *ybgF*), which enhance bacterial survival during exposure to stress or toxic unfolded proteins, by providing a mechanism for release of the unwanted periplasmic component (36–38). Interestingly, the σ^E controlled VrrA sRNA of *Vibrio cholera*, which is evolutionary unrelated to MicA/RybB, also regulates major two porins and controls OMV production (39, 40). Additional nonporin targets intermesh the σ^E response with other global regulatory systems. These include *phoP* (28), which monitors aspects of OM status; and possibly OmpR and σ^S through regulation of *ecnB* encoding a lipoprotein with a cell death phenotype (41). Finally, *rraB* and *rluD* are two RybB targets with a potential to globally affect the protein content of the cell. RraB binds to RNase E to alter endonucleolytic mRNA cleavage activity (42). RluD, a 23S rRNA pseudouridine synthase, is important for proper ribosome assembly function and biogenesis and affects translation termination (43, 44). Given these many targets, the exceptional strength of the *micA* and *rybB* promoters is likely necessary to continuously replenish the pool of the two sRNAs, because Hfq-dependent sRNAs are often codegraded with their mRNA targets (45).

Two network motifs warrant further study. First, three targets are both expressed by σ^E and down-regulated by the sRNAs. This creates the potential for an incoherent feed-forward loop, as σ^E can simultaneously provide positive and negative input to each target. Strikingly, two of these genes are deleterious when overexpressed, leading to cessation of growth (*yfeK*) or lysis (*htrG*; ref. 46). The incoherent feed-forward loop could prevent sRNA down-regulation of these genes implicated in cell death under severe conditions where homeostasis cannot be restored. Unconstrained σ^E activation could eliminate the most damaged cells, preventing them from competing for resources if growth resumed at a later time. Second, convergent target regulation by coactivated but unrelated sRNAs is unique (32). Both sRNAs recognize targets via conserved small GC-rich clusters near the 5′ end of the sRNA, but contact the mRNAs in disparate regions (e.g., *tsx* or *ompA*; Fig. 5), which validates the nonhomology of MicA and RybB. Other known cases of coinduced sRNAs use homologous sRNAs, which recognize the same region of the target mRNA, such as Qrr, OmrA and OmrB, or CyaR (5, 6, 47). It is possible that, under physiologically relevant conditions, MicA and RybB might need to partner for repression to achieve the optimal dosage. If so, this is an example of requiring concomitant activity of two coinduced regulators for target regulation. Whether σ^E-directed stress responses without predictable MicA/RybB homologs also use multiple unrelated sRNAs to create a repression arm remains an intriguing question.

The activation and repression arms of the σ^E response together ensure dynamic homeostatic control of the envelope compartment. Briefly, unassembled porins activate the DegS protease, which controls the rate of degradation of the σ^E antisigma, RseA (2). Because of extremely tight binding between RseA and σ^E, degradation of RseA is the predominant mechanism for generating free σ^E. Hence, the rate of RseA degradation sets σ^E activity (48). Rapid generation and degradation of sRNAs during the regulation process enables continuous adjustment of the flow of porin precursors to the envelope, enabling the cell to continuously adjust the activity of σ^E either upward or downward. The molecular dynamics of this control system are likely to be influenced by the kinetics of sRNA–target interactions and the growth-dependent occupancy of Hfq protein, as well as the distinct characteristics of the strong sRNA promoters themselves, which show differential activation in stationary phase (3).

There are intriguing similarities between the RyhB/Fur network that maintains Fe^{2+} homeostasis and the MicA/RybB/σ^E network. Fur is an active repressor in high Fe^{2+} conditions, but is inactive in low Fe^{2+} conditions. Hence, its repressed targets, including the RyhB sRNA, are expressed. The ~18 RyhB down-regulated target mRNAs are predominantly nonessential Fe^{2+}-containing proteins (49). Thus, target downregulation by *ryhB* effectively increases the Fe^{2+} pool so that Fur is again active as a repressor. In both networks, the sRNAs control a coherent set of mRNAs, providing a posttranscriptional repression mechanism as a counterpoint to transcriptional activation (or de-repression in the case of Fur). By influencing the signal controlling their respective transcription factors, both sRNAs intermesh the two arms of the response. The sRNA arms of both networks

seem essential during time of rapid change in the signal. This concordance suggests that temporal control may be a core aspect in constructing networks subject to extensive sRNA control, as has been argued in a recent kinetic analysis of mRNA regulation by CRP protein/Spot42 RNA (22). Given the extensive information now available for both the transcriptional and posttranscriptional events, the MicA/RybB/σ^E network will be an ideal test bed for understanding how hierarchical control and temporal differentiation are achieved in complex sRNA control systems.

Materials and Methods

Bacterial strains, plasmids, oligonucleotides, probes for hybridization, details of plasmid construction, and primers for qRT-PCR are provided in *SI Materials and Methods*. Cultures were grown in Luria Bertani (LB) or M9 complete minimal media as indicated. For a full description of materials and methods, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank M. Gao for technical assistance. This work was supported by the DFG Priority Program SPP1258 *Sensory and Regulatory RNAs in Prokaryotes* (DFG Grant Vo875/3-2; to J.V.), National Institutes of Health Grant RO1 GM036278-23 (to C.A.G.), and Training Grant T32 AI060537-07 (to E.B.G.).

1. Alon U (2007) Network motifs: theory and experimental approaches. *Nat Rev Genet* 8: 450–461.
2. Ades SE (2008) Regulation by destruction: design of the sigmaE envelope stress response. *Curr Opin Microbiol* 11:535–540.
3. Mutalik VK, Nonaka G, Ades SE, Rhodius VA, Gross CA (2009) Promoter strength properties of the complete sigma E regulon of Escherichia coli and Salmonella enterica. *J Bacteriol* 191:7279–7287.
4. Rhodius VA, Suh WC, Nonaka G, West J, Gross CA (2006) Conserved and variable functions of the sigmaE stress response in related genomes. *PLoS Biol* 4(1):43–59.
5. Gottesman S, Storz G (2010) Bacterial small RNA regulators: Versatile roles and rapidly evolving variations. *Cold Spring Harbor Perspectives Biol*, 10.1101/cshperspect.a003798.
6. Papenfort K, Vogel J (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe* 8:116–127.
7. Balbontín R, Fiorini F, Figueroa-Bossi N, Casadesús J, Bossi L (2010) Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in Salmonella enterica. *Mol Microbiol* 78:380–394.
8. Bossi L, Figueroa-Bossi N (2007) A small RNA downregulates LamB maltoporin in Salmonella. *Mol Microbiol* 65:799–810.
9. Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J (2008) Small RNA binding to 5′ mRNA coding region inhibits translational initiation. *Mol Cell* 32:827–837.
10. Johansen J, Rasmussen AA, Overgaard M, Valentin-Hansen P (2006) Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* 364:1–8.
11. Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J (2010) Evidence for an autonomous 5′ target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci USA* 107:20435–20440.
12. Papenfort K, et al. (2006) SigmaE-dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. *Mol Microbiol* 62: 1674–1688.
13. Rasmussen AA, et al. (2005) Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* 58:1421–1429.
14. Udekwu KI, et al. (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev* 19:2355–2366.
15. Udekwu KI, Wagner EG (2007) Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res* 35:1279–1288.
16. Guisbert E, Rhodius VA, Ahuja N, Witkin E, Gross CA (2007) Hfq modulates the sigmaE-mediated envelope stress response and the sigma32-mediated cytoplasmic stress response in Escherichia coli. *J Bacteriol* 189:1963–1973.
17. De Las Peñas A, Connolly L, Gross CA (1997) SigmaE is an essential sigma factor in Escherichia coli. *J Bacteriol* 179:6862–6864.
18. Button JE, Silhavy TJ, Ruiz N (2007) A suppressor of cell death caused by the loss of sigmaE downregulates extracytoplasmic stress responses and outer membrane vesicle production in Escherichia coli. *J Bacteriol* 189:1523–1530.
19. Hayden JD, Ades SE (2008) The extracytoplasmic stress factor, sigmaE, is required to maintain cell envelope integrity in Escherichia coli. *PloS One* 3:e1573.
20. Papenfort K, et al. (2009) Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. *Mol Microbiol* 74:139–158.
21. Thompson KM, Rhodius VA, Gottesman S (2007) SigmaE regulates and is regulated by a small RNA in Escherichia coli. *J Bacteriol* 189:4243–4256.
22. Beisel CL, Storz G (2011) The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in Escherichia coli. *Mol Cell* 41: 286–297.
23. Sharma CM, Vogel J (2009) Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol* 12:536–546.
24. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R (2004) Fast and effective prediction of microRNA/target duplexes. *RNA (New York, N. Y* 10:1507–1517.
25. Guillier M, Gottesman S, Storz G (2006) Modulating the outer membrane with small RNAs. *Genes Dev* 20:2338–2348.
26. Pfeiffer V, Papenfort K, Lucchini S, Hinton JC, Vogel J (2009) Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. *Nat Struct Mol Biol* 16:840–846.
27. Urban JH, Vogel J (2007) Translational control and target recognition by Escherichia coli small RNAs in vivo. *Nucleic Acids Res* 35:1018–1037.
28. Coornaert A, et al. (2010) MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol* 76:467–479.
29. Rhodius VA, Mutalik VK (2010) Predicting strength and function for promoters of the Escherichia coli alternative sigma factor, sigmaE. *Proc Natl Acad Sci USA* 107: 2854–2859.
30. Ruiz N, Kahne D, Silhavy TJ (2009) Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nat Rev Microbiol* 7:677–683.
31. Silhavy TJ, Kahne D, Walker S (2010) The bacterial cell envelope. *Cold Spring Harb Perspect Biol* 2:a000414.
32. Beisel CL, Storz G (2010) Base pairing small RNAs and their roles in global regulatory networks. *FEMS Microbiol Rev* 34:866–882.
33. Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8:519–529.
34. Mecsas J, Rouviere PE, Erickson JW, Donohue TJ, Gross CA (1993) The activity of sigma E, an Escherichia coli heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev* 7(12B):2618–2628.
35. Mecsas J, Welch R, Erickson JW, Gross CA (1995) Identification and characterization of an outer membrane protein, OmpX, in Escherichia coli that is homologous to a family of outer membrane proteins including Ail of Yersinia enterocolitica. *J Bacteriol* 177: 799–804.
36. Kuehn MJ, Kesty NC (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev* 19:2645–2655.
37. McBroom AJ, Johnson AP, Vemulapalli S, Kuehn MJ (2006) Outer membrane vesicle production by Escherichia coli is independent of membrane instability. *J Bacteriol* 188: 5385–5392.
38. Xia XX, Han MJ, Lee SY, Yoo JS (2008) Comparison of the extracellular proteomes of Escherichia coli B and K-12 strains during high cell density cultivation. *Proteomics* 8: 2089–2103.
39. Song T, et al. (2008) A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles. *Mol Microbiol* 70:100–111.
40. Song T, Sabharwal D, Wai SN (2010) VrrA mediates Hfq-dependent regulation of OmpT synthesis in Vibrio cholerae. *J Mol Biol* 400:682–688.
41. Bishop RE, Leskiw BK, Hodges RS, Kay CM, Weiner JH (1998) The entericidin locus of Escherichia coli and its implications for programmed bacterial cell death. *J Mol Biol* 280:583–596.
42. Gao J, et al. (2006) Differential modulation of E. coli mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol Microbiol* 61:394–406.
43. Ejby M, Sørensen MA, Pedersen S (2007) Pseudouridylation of helix 69 of 23S rRNA is necessary for an effective translation termination. *Proc Natl Acad Sci USA* 104: 19410–19415.
44. Gutgsell NS, Deutscher MP, Ofengand J (2005) The pseudouridine synthase RluD is required for normal ribosome assembly and function in Escherichia coli. *RNA (New York, N. Y)* 11:1141–1152.
45. Massé E, Escorcia FE, Gottesman S (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli. *Genes Dev* 17(19):2374–2383.
46. Asakura Y, Kobayashi I (2009) From damaged genome to cell surface: transcriptome changes during bacterial cell death triggered by loss of a restriction-modification gene complex. *Nucleic Acids Res* 37:3021–3031.
47. Svenningsen SL, Tu KC, Bassler BL (2009) Gene dosage compensation calibrates four regulatory RNAs to control Vibrio cholerae quorum sensing. *EMBO J* 28:429–439.
48. Grigorova IL, et al. (2004) Fine-tuning of the Escherichia coli sigmaE envelope stress response relies on multiple mechanisms to inhibit signal-independent proteolysis of the transmembrane anti-sigma factor, RseA. *Genes Dev* 18:2686–2697.
49. Massé E, Vanderpool CK, Gottesman S (2005) Effect of RyhB small RNA on global iron use in Escherichia coli. *J Bacteriol* 187:6962–6971.