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Role of sRNAs in the sE Dependent Cell Envelope Stress Response in E.coli

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Role of sRNAs in the σ^E Dependent Cell Envelope Stress Response in *E.coli*

by

Emily Gogol

DISSERTATION

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GRADUATE DIVISION

[redacted]

Dedication

To my family and friends.

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Chapter 2 and chapter 3 contain data that is currently unpublished. E.B.G., C.A.G., V.A.R., G.S. designed the research. E.B.G. performed all experiments, analyzed data, and contributed new reagents/analytical tools

Role of sRNAs in the σ^E Dependent Cell Envelope Stress Response in *E.coli*

Emily Gogol

The cell envelope in gram-negative bacteria is a specialized barrier that must be constantly remodeled to suit the bacteria's needs. To accomplish this end bacteria have numerous systems in place that monitor, repair, and reconfigure their cell envelope as needed. One of the most critical systems in *E.coli* that participates in this function is the σ^E dependent cell envelope stress response. Although much is known about the protein encoded components of this critical system, little was known about the components that encode for small regulatory RNAs (sRNAs).

This work clearly establishes sRNAs as a central component of the σ^E dependent cell envelope stress response. We uncovered many new σ^E dependent sRNAs, including a novel sRNA, Reg26, which shares some characteristics with the other previously known σ^E dependent sRNAs MicA and RybB. Not only does this work fully characterize the regulatory breadth of MicA, RybB, and Reg26, it characterizes the importance of these sRNAs by assessing their contributions to cell survival. Overall this work demonstrates how these sRNAs, in concert with the protein encoded component of the response, enables σ^E to monitor and maintain a trait as complex as envelope homeostasis.

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INTRODUCTION

Bacterial sRNAs are powerful effectors in an array of processes ranging from quorum sensing to membrane homeostasis, and we are beginning to understand how they bring about rapid, and often widespread, change. All known trans-encoded anti-sense sRNAs act by base-pairing to a target mRNA, and in this way alter how the mRNA is processed by the cell. This kind of RNA-RNA interaction is dependent upon the presence of the RNA binding protein Hfq. Thus, when the outcome of a response is found to be dependent upon Hfq, it suggests that at least one sRNA is critical to that response. Our lab found that the envelope stress response in *E. coli* is dependent on Hfq, which strongly suggested that σ^E regulated sRNAs participate in the envelope stress response. Specifically, the rapid mRNA downregulation of seven outer membrane proteins (OMPs) and one lipoprotein observed upon σ^E overexpression is abolished in strains lacking Hfq. Many of the downregulated mRNAs code for porins, that when misfolded, activate σ^E, producing a homeostatic regulatory loop. Other labs have identified two σ^E controlled sRNAs that contribute to the downregulation of some porin mRNA. However, the sRNAs downregulating the remainder, and the role of σ^E regulated sRNAs in establishing the kinetics and dynamics of the σ^E -mediated response remains to be determined. To investigate the theory that sRNAs provide essential regulation of the outer membrane and control aspects of the envelope stress response is the goal of this study.

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 as 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) (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 non-stress 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. Promoters recognized by σ^E have been mapped to saturation in *E. coli*, revealing that σ^E directly activates ~60 transcriptional units which 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 post-transcriptionally. Intriguingly, the distribution of promoter strengths in the σ^E regulon suggests candidates for such repressors, 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).

The central role of the σ^E response is to ensure that the protein components of the OM are folded and present in correct ratio to the LPS. Both arms of the response collaborate to maintain folding homeostasis for OM B-barrel proteins. In response to the accumulation of unfolded B-barrel proteins, expression of the chaperone apparatus and machinery necessary for B-barrel protein insertion into the membrane are upregulated, as well as the central component of the LPS insertion machinery, itself a B-barrel protein (4). The sRNA arm of the response complements this slow response by immediately downregulating expression of porin mRNAs to decrease the flow of unassembled porins into the envelope (4, 8, 12, 14, 16, 17). Previous work in *S. typhimurium* pointed to RybB as a major regulator of many porin mRNAs (12). This work suggested that by utilizing sRNAs σ^E provides a regulatory mechanism that allows for the cell to adjust the flow of porins relative to the assembly capacity of the cell.

The production of outer membrane vesicles (OMVs) constitutes a second mechanism for relieving OM stress. OMVs are spherical blebs of the outer membrane that contain periplasmic proteins, peptidoglycan (PG) and LPS. Production of OMVs enhance bacterial survival during exposure to stress or toxic unfolded proteins, as they

provide a mechanism for release of unwanted periplasmic components, as well has having roles in pathogenesis and cell-cell communication (18-20). Previously, McBroom and Kuehn 2006 have identified gene deletions that enhance OMV production. Strikingly, many mRNAs downregulated by the sRNA arm of the σ^E response correspond to functions identified by this analysis, suggesting that this arm of the response promotes OMV production. First, lack of OMPs favors OMV formation, and the sRNA arm reinforces a dramatic decrease in porin insertion in the OM. Second, reducing cross-links between the PG and OM is believed to serve as sites of nucleation for OMV formation; YcsF, the transpeptidase responsible for covalent attachment of peptidoglycan to the outer membrane (21, 22), is downregulated by MicA, thereby facilitating the formation of OMVs. MicA downregulation of Ycfs provides a mechanistic explanation for the observation that a Δ*rpoE* strain has increased levels of YcfS (23). Finally, a deficit in Pal also promotes OMV formation; both Pal and YbgF (a Tol-Pal interacting protein) are also downregulated (24). Interestingly, Pal and YbgF are members of the target class where high overexpression of σ^E ameliorates downregulation, suggesting that during later stages of the response downregulation is neutralized. Taken together, these results suggest that OMV production is a second mechanism by which the sRNA arm of the response counteracts folding limitations induced by a deficit of chaperones and insertion machinery.

The sRNA arm of the response also intermeshes the σ^E response with that of other global regulatory systems to provide an integrated response. It had already been evident that the sRNA arm links the σ^E reponse to the many other sRNA responses altering porin flux (25, 26). Here, we report that the MicA sRNA mediates a direct connection to the

PhoPQ two component regulatory system, which activates genes involved in Mg++ homeostasis, resistance to antimicrobial peptides and LPS modification in response to low levels of divalent cations (27-29). In addition, recent work by Cooneart et al. establishes that MicA downregulates PhoP mRNA, thereby linking expression of the PhoP transcriptional regulator to status of the σ^E response (30). MicA also collaborates with PhoP on another level by downregulating LpxT, which mediates an Lps modification (*lpxT*). MicA also provides a direct connection to an important output of OmpR and σ^E responses by downregulating EcnB, a toxic lipoprotein. EcnB has a cell death phenotype during high osmolarity, stationary phase growth, conditions that alleviate OmpR repression and promote σ^S activation of *ecnB* (31). As σ^E is also active under such conditions, it serves to partially counteract σ^S activation of *ecnB*. Finally, two RybB targets, *rraB* and *rluD*, have the potential to globally affect the protein content of the cell. RraB binds to RNase E, altering the cleavage targets of RNase E (32). RluD, a 23S rRNA pseudouridine synthase, is important for proper ribosome assembly, function and biogenesis (33)and affects the termination of translation by the ribosome (34).

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 results in novel convergent target regulation by bacterial sRNAs. These two noncoding regulators constitute a post-transcriptional 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. Moreover, we uncover a suite of novel sRNAs that are also regulated by σ^E. One of these new sRNAs, Reg26 also plays an important role in the σ^E response. Reg26 targets the mRNA of an important constituent of the cell envelope, *Lpp*, for downregulation. We also demonstrate that without Reg26 the cell suffers from growth defects that result in a bacteriostatic phenotype.

CHAPTER 1

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

I. Background

The *E. 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 non-coding 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 post-transcriptional 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 non-porin targets predict roles for MicA and RybB in crosstalk with other regulatory responses. This work also provides the first example of evolutionarily unrelated sRNAs that are co-induced 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 post-transcriptional 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.

II. Results and Discussion

The quality control functions of MicA and RybB are central to the σ^E response

Lack of σ^E is lethal to *E. coli* (35). 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 (36, 37). To evaluate the physiological impact of MicA and RybB, we utilized 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 decreases (Fig. 2), as previously reported (37). Importantly, concomitant overexpression of either MicA or RybB rescues both growth and viability phenotypes exhibited following σ^E shutoff (Fig. 2A-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. 2C). 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. 2C), 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 4 different conditions to

accommodate the possibility that regulation was growth-phase specific (exponential vs stationary phase) as noted previously for the Hfq-associated ArcZ (38), or media specific (glucose vs maltose) (columns 1-6 of Table S1, Table S2, Fig. S1A).

This identified 31 regulated mRNAs, all of which were negatively regulated; 80% responded in at least three conditions, while ~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 includes 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) (9). As previously observed (16) the *yhcN* and *lpp* mRNAs gave a very small (≤2-fold) change to both σ^E or sRNA over-expression, suggesting little regulation, or regulation taking place solely at the level of translation (Fig. S1C). MicA and RybB not only downregulate 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.

We determined whether targets were also repressed by overexpression of σ^E, as expected because this condition induces chromosomal MicA and RybB (12, 17, 39). Many targets (15) were significantly repressed under this condition (column 2 of Table S1), but 3 targets were upregulated (*htrG*, *yfeK* and *yhjJ*). All three genes have upstream σ^E-dependent promoters (4), leading to net upregulation 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 when regulation is primarily translational changes in mRNA levels are below the threshold for significance in microarray studies (40, 41). Alternatively, compensatory activities in the regulon may mask repression**.** A timecourse of σ^E overexpression for select targets indicated some temporal distinction in the dynamics of mRNA level changes (Fig. S2). For example, *nmpC* was 4-fold repressed after 5' of σ^E overexpression, which is 50% of the repression level observed after 20' of σ^E overexpression. In contrast, *ompF* exhibited little repression at 5' but was repressed 64-fold after 20' of σ^E overexpression.

The 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 (42) predicts that most newly discovered RybB targets are also guided by R16 (Fig. S3). We therefore 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->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, 43). Of the 17 candidate targets predicted to utilize the R16 region of RybB 16/17 are significantly downregulated by R16TOM (exception *ydeN*; Fig. 3A).

Moreover, RybB-M2 (C_2->G change) is unable to downregulate13/14 targets predicted to utilize RybB C_2 for interaction (exception *ompW*) whereas 3/3 targets predicted not to utilize mutation RybB C_2 (*lamB*, *fimA*, *ydeN*) maintain downregulation (Fig. 3A). 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 co-expressed with a translational fusion of the target 5' mRNA region to green fluorescent protein (GFP) (44). This assay validated *E. coli nmpC*, *ompC*, and *ompF* as direct RybB targets (Fig. S4). Note that our data revises a previously proposed RybB-*ompC* pairing (39), 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. 3B-C), encoding respectively a catecholate siderophore receptor in the OM, and a conserved cytosolic 23S rRNA pseudouridine synthase. 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 basepairing (G->C change at positions -71 (Fig. 3B) or +4 (Fig. 3C) 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. 4A), we constructed a series of MicA

truncations/mutations (schematized in Fig. 4A), and examined their repression capacity to identify critical features of MicA. All 10 targets tested were downregulated by the highly conserved nucleotides 1-24 and half were also downregulated by nucleotides 8-24 (Fig. 4B; 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 (30).

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. Downregulation was abrogated by mutational change when pairing was predicted (6/6 for C_7->G; 10/10 for C_{11}->G; Fig. 4C), and maintained for all 4 targets predicted not to utilize C_7 (Fig. 4C, 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. 4B), 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 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. 4D). Using an *ompX::gfp* fusion and a compensatory M7' allele, we demonstrated that C_7 is essential for the repression of this target (Fig. 4E), which gives the first clue as to why the 5' terminal positions of MicA are highly conserved (Fig. 4A).

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. 5A), 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. 5B). 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. 5C). Notably, a mutation in the MicA binding site has no effect on the ability of RybB to interact with *tsx* mRNA (Fig. 5D: 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. 5C) 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* of was regulated by neither RybB-M2 nor MicA (Fig. 5E). 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*.

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 (45, 46), whereas, as described below, the repression arm alleviates stress and interconnects regulatory networks. 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 (47). 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 upregulate protein folding chaperones and catalysts; simultaneously, protein synthesis is downregulated by a separate pathway to stem the flow of precursors into the ER (48).

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 (49, 50). 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 (18-20). 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 (51, 52). Additional non-porin targets intermesh the σ^E response with other global regulatory systems. These include *phoP* (30), which monitors aspects of OM status; and possibly OmpR and σ^S through regulation of *ecnB* encoding a lipoprotein with a cell death phenotype (31). 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 (32). RluD, a 23S rRNA pseudouridine synthase, is important for proper ribosome assembly function and biogenesis and affects translation termination (33, 34). 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, since Hfq-dependent sRNAs are often co-degraded with their mRNA targets (53).

Two network motifs warrant further study. First, three targets are both transcribed by σ^E and downregulated 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*) (54). The incoherent feed forward loop could prevent sRNA downregulation of these genes implicated in cell death under severe conditions where homeostasis cannot be restored (55). Unconstrained σ^E activation could eliminate the most damaged cells, preventing them competing for resources if growth resumed at a later time. Second, convergent target regulation by co-activated but unrelated sRNAs is novel (47). 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 non-homology of MicA and RybB. Other known cases of co-induced sRNAs utilize homologous sRNAs which recognize the same region of the target mRNA, such as Qrr, OmrAB, or CyaR (5, 6, 56). 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 the first example of requiring concomitant activity of two co-induced regulators for target regulation. Whether σ^E-directed stress responses without predictable MicA/RybB homologues 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 (57). 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 the Fur/RyhB network that maintains Fe^{++} homeostasis and the MicA/RybB/σ^E network. Fur is an active repressor in high Fe^{++} conditions, but is inactive in low Fe^{++} conditions. Hence, its repressed targets, including the RyhB sRNA are expressed. The ~18 RyhB downregulated target mRNAs are predominantly nonessential Fe^{++}-containing proteins (58). This effectively increases the Fe^{++} pool so that Fur is again active as a repressor. In both networks, the sRNAs control a coherent set of mRNAs, providing a post-transcriptional 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 (40). Given the extensive information now available for both the transcriptional and post-transcriptional 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.

III. Figures

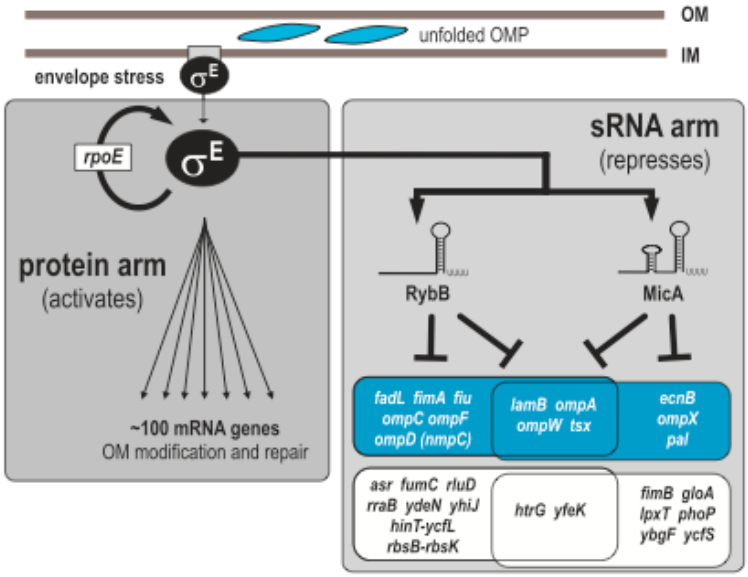

Figure 1. Initiation of the σ^E response and its immediate effects

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Illustration of 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 downregulation 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.

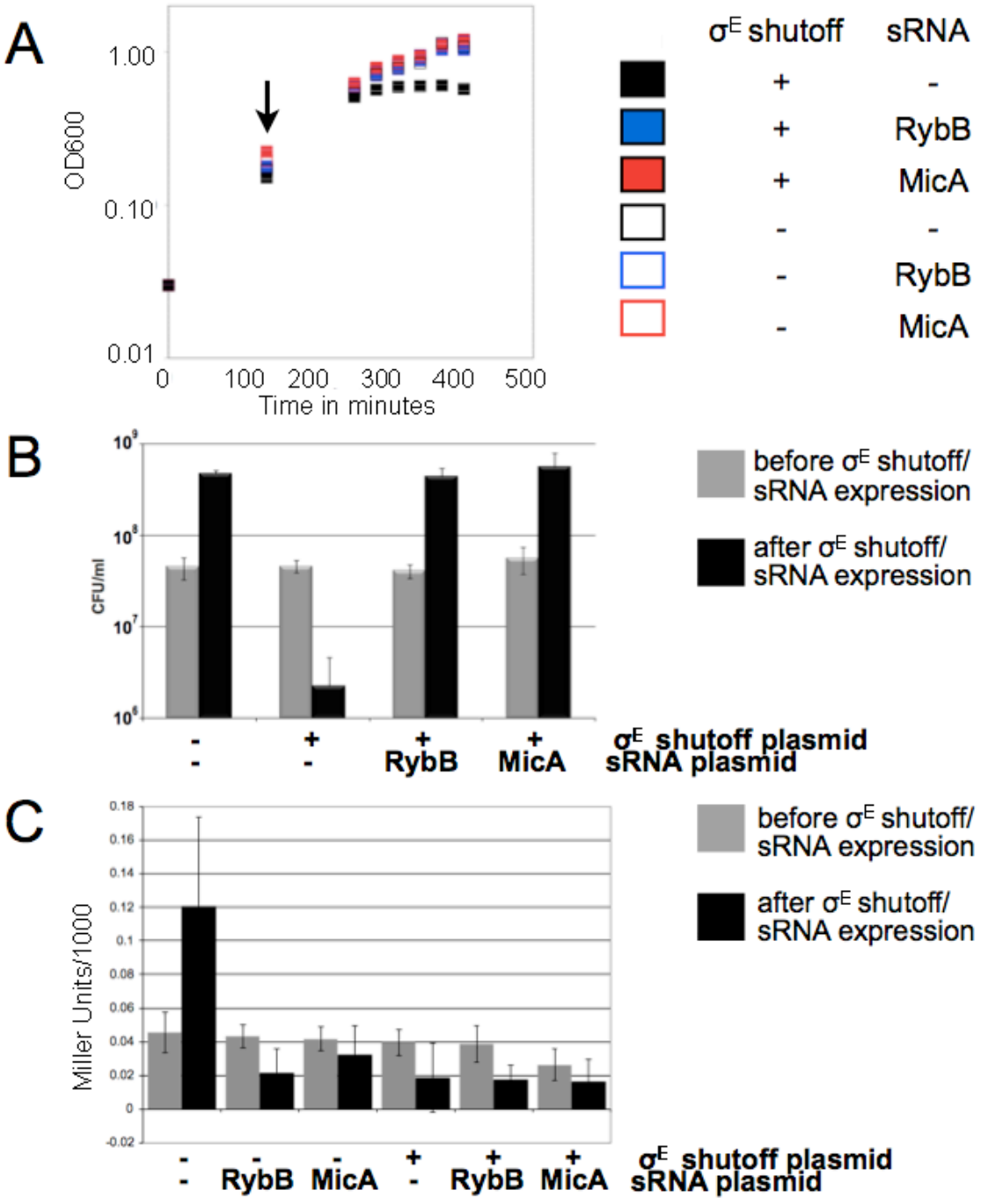

Figure 2. RybB and MicA protect cells from lysis during σ^E shutoff

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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 ($+\sigma^E$ 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 ($+\sigma^E$ shutoff, - sRNA plasmid) showed reduced colony forming units following σ^E shutoff; concomitant overexpression of either sRNA ($+\sigma^E$ shutoff, RybB; $+\sigma^E$ shutoff, MicA) fully restored viability; (C) σ^E activity of each strain shown in (A) both prior to 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 OD600 = 0.03 in fresh media and grown at 30°C. 1mM IPTG was added just before 135 minutes of growth (OD600 ~0.1) to induce overexpression of RseA/B, MicA and RybB, as indicated by the arrow. The "-" sample was taken just prior to 135 minutes of growth and the "+" sample was taken at 255 minutes of growth. The average of three experiments with SD is shown.

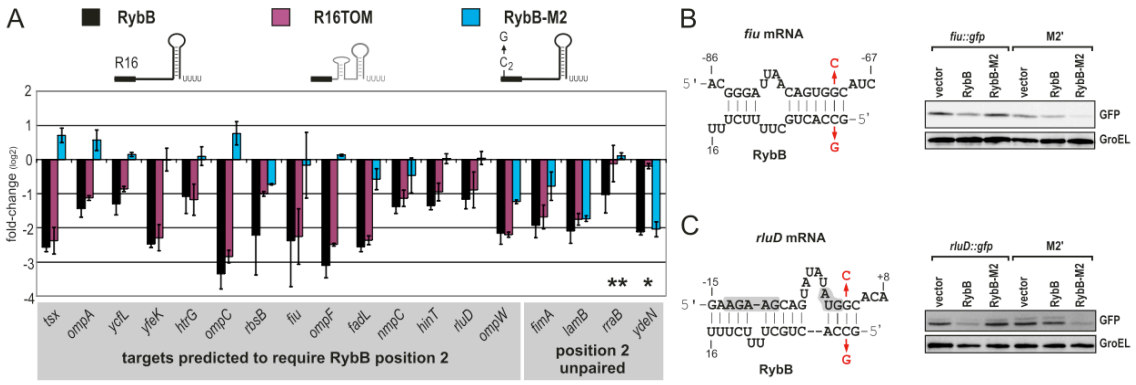

Figure 3. Region and nucleotide specific binding by RybB

Figure 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 is the average of three experiments with SD. ** indicates R16TOM should not be sufficient to regulate *rraB*, and * indicates R16TOM should be sufficient to regulate *ydeN*. A schematic of interaction map and the mutations used for validation are depicted in the left panel of Fig. 3B (*fiu*) and 3C (*rluD*). Experimental results from *gfp* translational reporters monitored by Western blot are indicated in the right hand panels of 4B,C. Bacterial growth, induction, qRT-PCR and analysis as described in Materials and Methods.

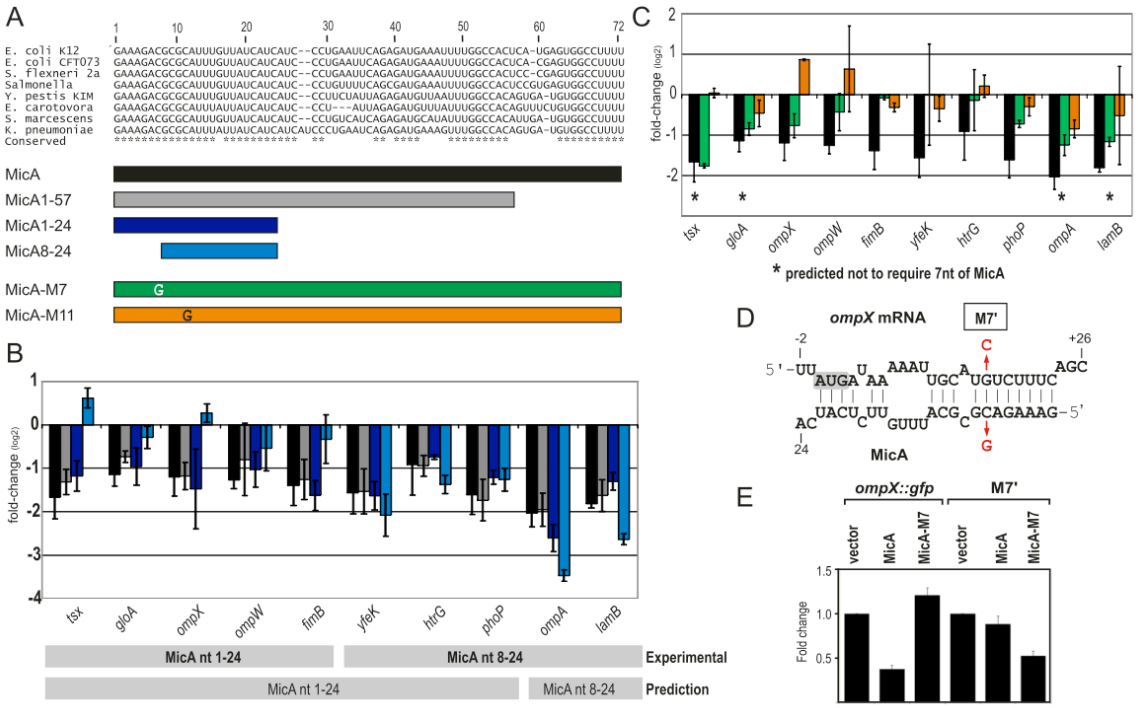

Figure 4. Region and nucleotide specific binding by MicA

Figure 4. Region and nucleotide specific binding by MicA

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

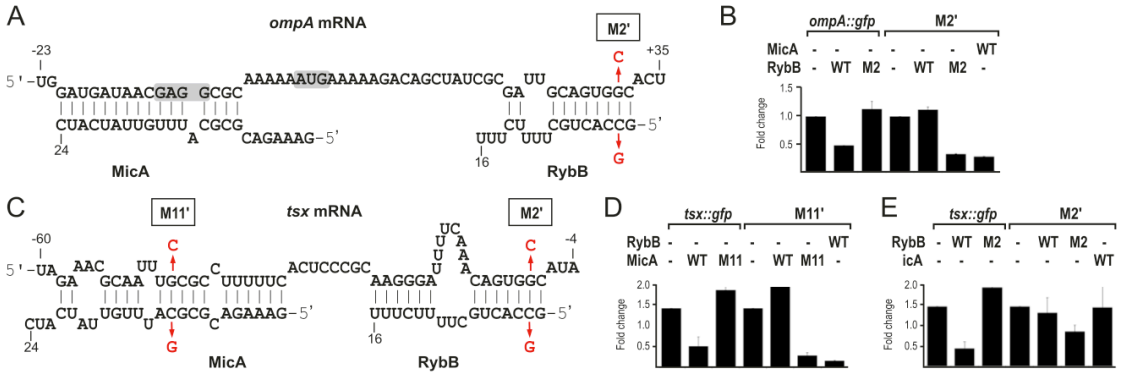

Figure 5. Convergent target regulation by MicA and RybB

Figure 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 employed for validation are shown in Fig. 5A (*ompA*) and 5C (*tsx*). Results from *gfp* translational reporter assays employing these constructs performed as described in Fig. 4E is shown in Fig. 5B (RybB/*ompA*), 5D (MicA/*tsx*) and 5E (RybB/*tsx*).

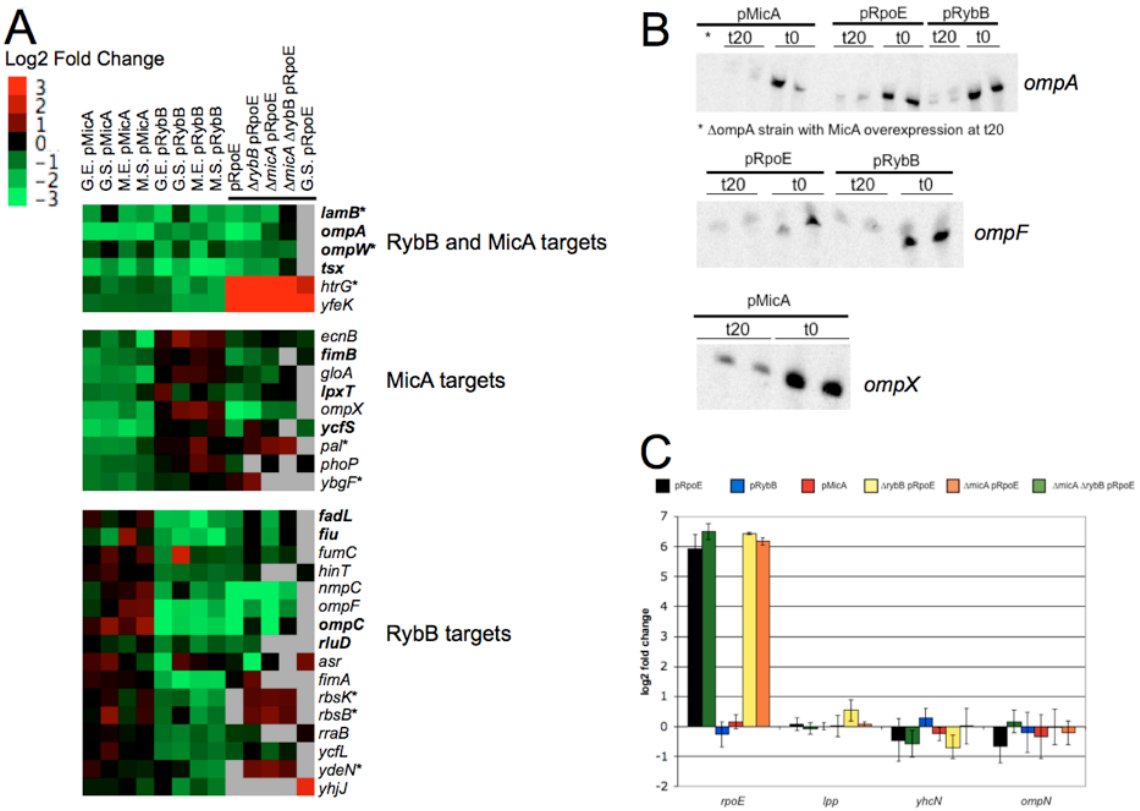

Figure S1. MicA and RybB regulate a variety of new targets

Figure S1. MicA and RybB regulate a variety of new targets

Shown is the response of specific mRNAs to overexpression of either σ^E (pRpoE), RybB (pRybB), MicA (pMicA), or σ^E overexpression in a delta RybB (Δ*rybB* pRpoE), delta MicA (Δ*micA* pRpoE) or delta MicA RybB strains (Δ*micA* Δ*rybB* pRpoE).

(A) Microarray (non-underlined genotypes) and qRT-PCR (underlined genotypes) data of the mRNA targets of MicA and RybB in response to a variety of growth conditions. Targets in bold indicate that regulation by the indicated sRNA is necessary and sufficient for σ^E dependent regulation. Bacteria grown overnight at 30˚C in glucose minimal media were diluted to OD_{450} = 0.03 in fresh minimal media with glucose (G) or maltose (M) and grown at 30°C to either mid-exponential phase (E; OD_{450} = 0.3) or early stationary phase (S; OD_{450} = 0.9). At this point, a pre-induction (time=0) sample was harvested, cultures were induced with 1mM IPTG, and a 20 min. post-induction sample was harvested (time=20'). Gray boxes indicate no data available. Microarray analysis was performed in 4 media (G.E., G.S., M.E. M.S.); follow-up qRT-PCR analysis was in G.E. and G.S. unless target is noted with an * which indicates exponential phase data from M.E. (B) Northern blots showing sRNA regulation of *ompA*, *ompF* and *ompX* mRNA; 5ug of RNA was loaded per lane.RNA was prepared and transcript abundance assayed by Northern Blot. qRT-PCR data in (C) is to show that there is equal overexpression of *rpoE* in pRpoE strains and the mRNA abundance of genes that are not significantly regulated by *rpoE*, MicA or RybB overexpression. cDNA was prepared and transcript abundance assayed by qRT-PCR as described in SI text. Transcript abundance of each mRNA was quantified relative to time=0 of its own genotype and are plotted as $\log_2$ fold change (time 20/time 0).

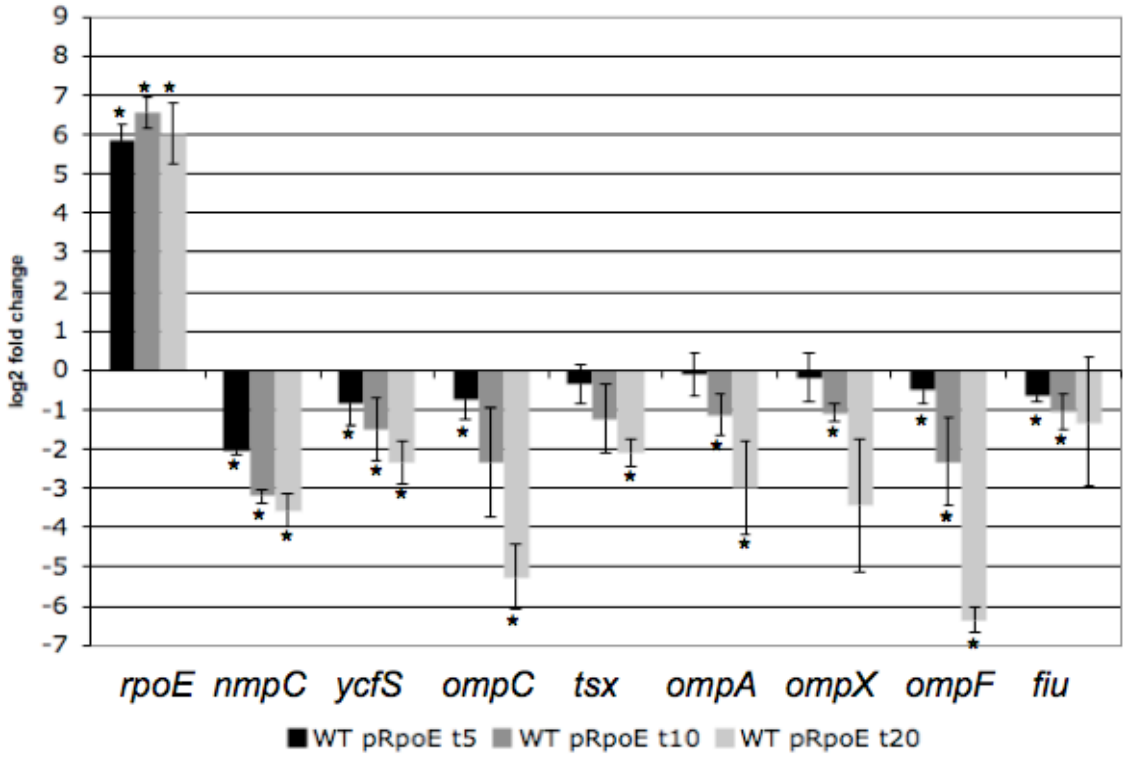

Figure S2. qRT-PCR of targets at 5, 10 and 20 minutes after overexpression of σ^E

Figure S2. qRT-PCR of targets at 5, 10 and 20 minutes after overexpression of σ^E

Bacteria grown overnight in minimal media were subcultured to OD_{450} = 0.03 in fresh minimal media with glucose and grown at 30°C to an OD_{450} of 0.3, and a pre-induction ('time 0') sample taken. Cultures were then induced with 1mM IPTG and 5, 10 and 20 minutes post-induction samples taken (t5, t10, t20). The average of three experiments with standard deviations is shown and data marked with an * indicate a $p < 0.01$. Transcript abundance was calculated as described for Figure S1.

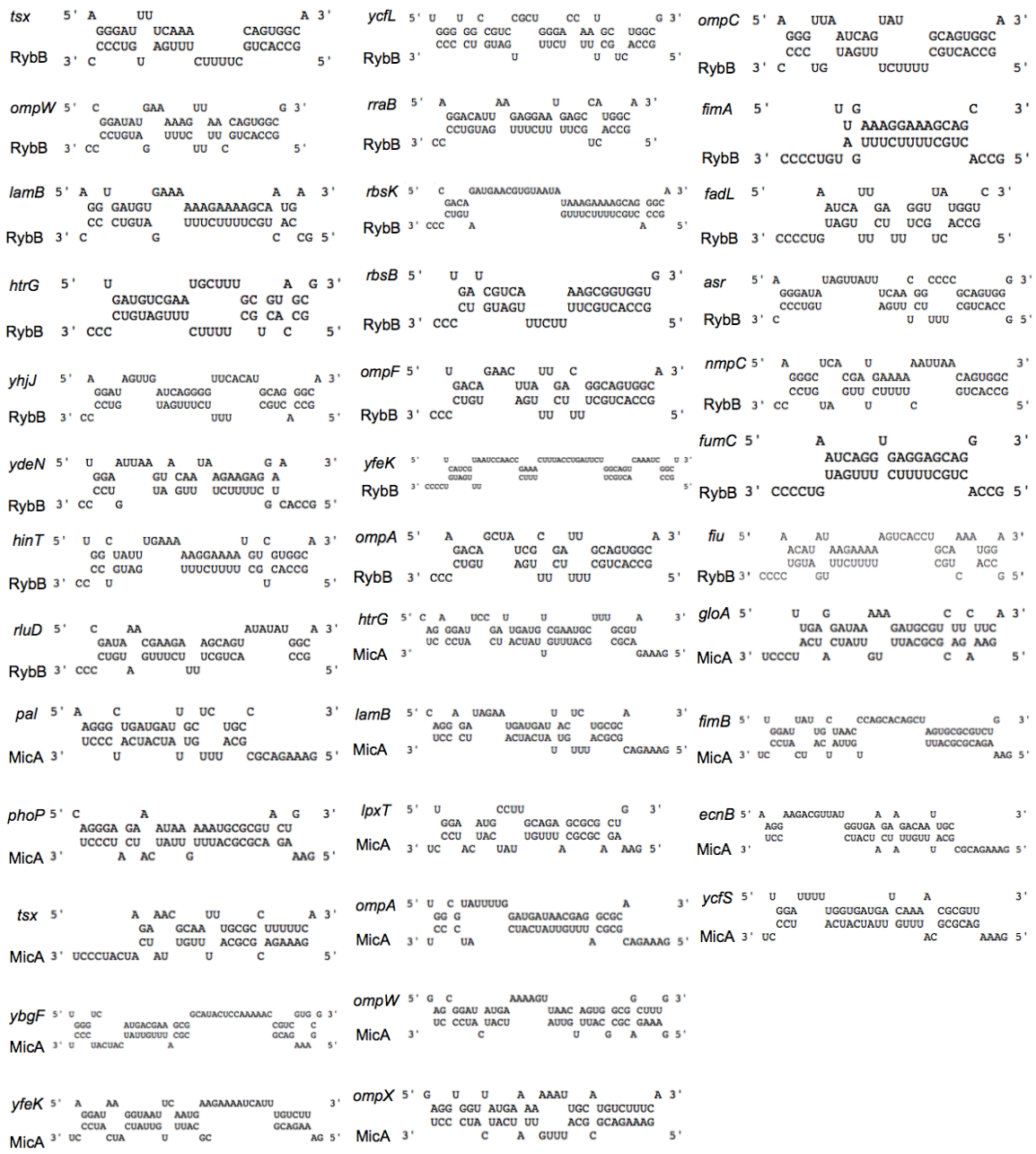

Figure S3. Predicted Interactions between MicA, RybB, and their target mRNAs

Figure S3. Predicted Interactions between MicA, RybB, and their target mRNAs

The freely available software RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) was used to predict alignments between MicA, RybB, and their targets, using the default parameters. For MicA, the 5' end 1-30nt was used, and for RybB, the 5' end 1-25nt except for fadL which used 1-16nt. The regions were chosen as our study showed they are sufficient for almost all target regulation (see Figure 3). Roughly from -100 from the start of translation to +20 was used for all targets, with *ompW*, *ydeN*, and *pal* through to +40. Start and stop information is as follows: lamB(RybB -41,-14)(MicA -11,+19); ompA(RybB +8,+34)(MicA -33,-5); ompW(RybB -5,+21)(MicA -8,27); tsx(RybB -6,-25)(MicA -59,-34); htrG(RybB -76,-52)(MicA -90,-55); yfeK(RybB -77,-27)(MicA -16,+36); ecnB(MicA -12,+20); fimB(MicA -57,-19); gloA(MicA -14,+15); lpxT(MicA -56,-32); ompX(MicA -9,+24); ycfS(MicA -10,+19); pal(MicA +22,+44); phoP(MicA -15,+12); ybgF(MicA -65,-24); fadL(RybB -13,+6) fiu(RybB -27,+5) fumC(RybB -3,-20); hinT(RybB -25,+6); rluD(RybB +6,-23); asr(RybB -39,-5); fimA(RybB -16,-1); rbsK(RybB -81,-45); rbsB(RybB -83,-63); rraB(RybB -22,+6); ycfL(RybB -49,-17); ydeN(RybB +2,+28); yhjJ(RybB -23,+11)

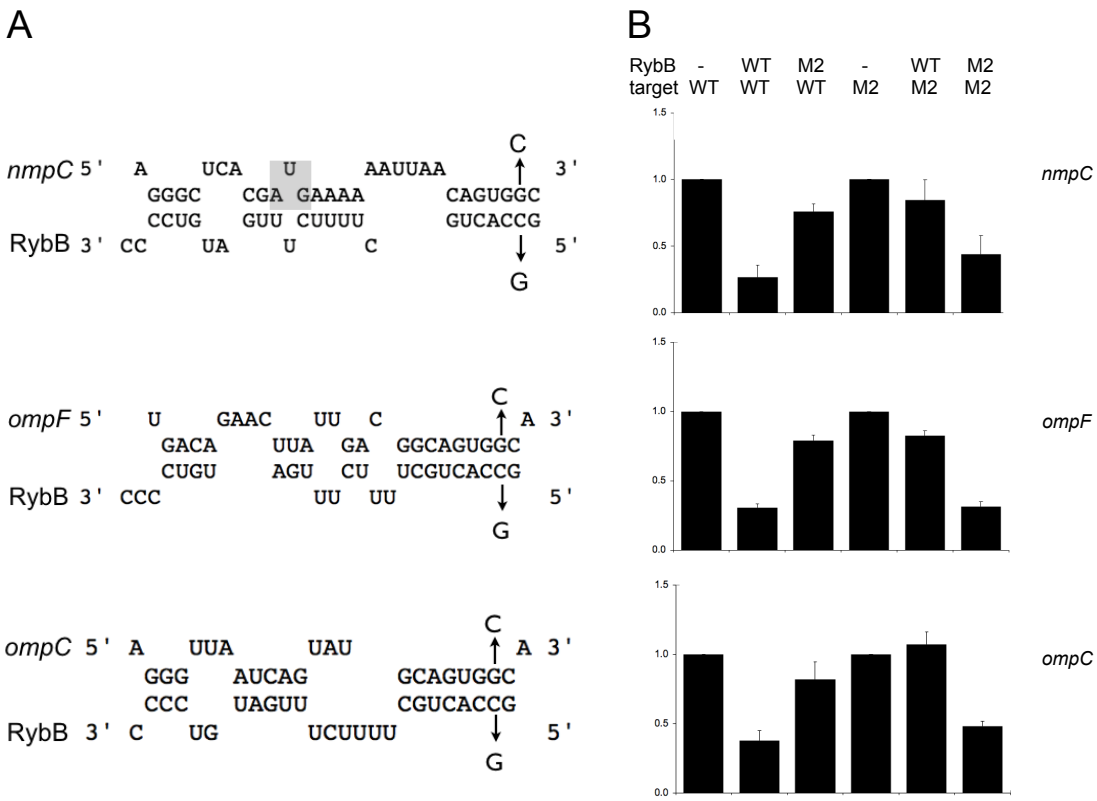

Figure S4. Expression characteristics of additional *target-gfp* fusions

Figure S4. Expression characteristics of additional *target-gfp* fusions

Shown is the regulation of select mRNAs targeted by RybB as indicated in the (A) interaction map with corresponding mutations. If present, the start codon is highlighted in grey. The framing nucleotides for each target interaction are as follows: *nmpC* (+20 to -10) *ompF* (-36 to -51) and *ompC* (-42 to -64). (B) Validation of *nmpC, ompF* and *ompC* as RybB targets using *gfp* reporters. All strains utilized in these experiments contain 2 plasmids, one for expressing the target –GFP fusion and the other for expressing the sRNA. "-" indicates that the strain has parent (control) plasmid that does not express sRNA; "WT", "M2", etc., indicate the sRNA variant expressed by the plasmid. For fold change calculations, GFP fluorescence of strain expressing only the target-GFP fusion is set at 1. Fold-change indicates ratio of GFP fluorescence of (strain expressing both sRNA and target / strain expressing target only). The average of three experiments with SD is shown.

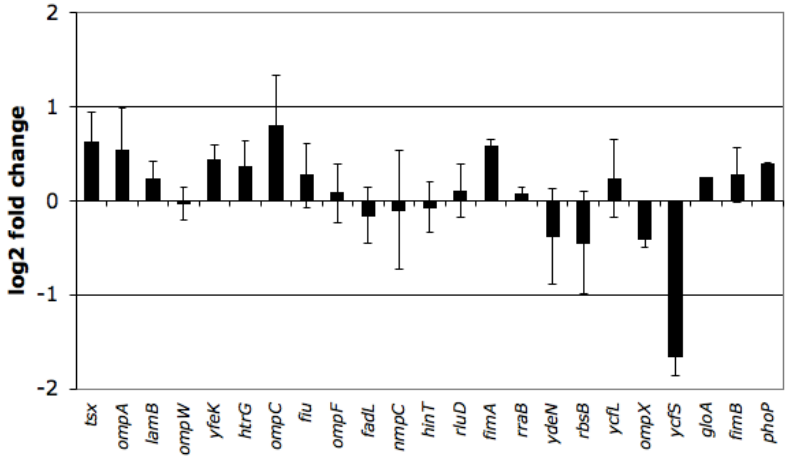

Figure S5. OmrB regulation of target mRNA

Figure S5. OmrB regulation of target mRNA

Regulation of specific targets after expression of the OmrB only fusion construct (see Fig. 3 and Fig. 4 for diagrams of fusion constructs to RybB and MicA). Here, only the OmrB backbone was expressed to determine if OmrB without MicA or RybB regulated any of the MicA or RybB target mRNAs. Only *ycsF* is downregulated significantly (≥2-fold). Growth conditions, target regulation criteria, and methods are the same as for Fig. 3 and Fig. 4.

Table S1 Summary of target interaction data for MicA and RybB

Target	σ^E regulation[a]	Type of regulation[b]	Log2 fold-change[c]		Condition[d]	sRNA	Target mRNA interaction region[e]	Sufficient minimal truncation[f]		Point Mutation[b]			Compensatory Mutation[i]	Target Description
			pMicA	pRybB				Fragment	Predicted[g]	Position	Abrogate	Predicted	Made/Restored	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A. Joint Targets of MicA and RybB														
lamB	-	N/S	-1.9[q]	-2.1[q]	GE ME MS	RybB	B1	1-16	Y	2	N	N		maltose outer membrane porin (maltoporin)
						MicA	B2	8-24	Y	7	N	N		
										11	N	Y		
ompA	-	N/S	-2.6	-1.2	GE GS ME MS	RybB	C	1-16	Y	2	Y	Y	Y/Y	outer membrane protein A
						MicA	B2	8-24	Y	7	N	N		
										11	Y	Y	footnote[i]	
ompW	-	N/S[k]	-1.1[q]	-1.9[q]	GE[p] ME	RybB	B2	1-16	Y	2	N	Y		outer membrane protein, receptor for Colicin S4
						MicA	B2	1-24	Y	7	N	Y		
										11	Y	Y		
tsx	-	N/S	-2.3	-3.2	GE GS ME MS	RybB	B2	1-16	Y	2	Y	Y	Y/N[e]	nucleoside channel, receptor of phage T6 and colicin K
						MicA	A	1-24	Y	7	N	N		
										11	Y	Y	Y/Y	
htrG	+	U	-1.1[r]	-2.1[r]	GE GS ME MS[o]	RybB	A	1-16	Y	2	Y	Y		predicted signal transduction protein (SH3 domain)
						MicA	A	8-24	N	7	Y	Y		
										11	Y	Y		
yfeK	+	U	-0.9	-1.6[q]	GE GS ME MS	RybB	A	1-16	Y	2	Y	Y		predicted protein
						MicA	B2	8-24	N	7	Y	Y		
										11	Y	Y		
B. Targets of MicA														
ecnB	-	S	-2.7[a]	0.3	GS MS	MicA	B2	n.d.	n.d.	7	n.d.	n.d.		enterocidin B membrane lipoprotein
										11	n.d.	n.d.		
fimB	-	N/S	-1.6	0.2	GE GS ME MS	MicA	B1	1-24	Y	7	Y	Y		tyrosine recombinase/inversion of on/off regulator of fimA
										11	Y	Y		
gloA	none	S[o]	-1.5	-0.1	GE GS ME MS	MicA	B2	1-24	Y	7	N	N		glyoxalase I, Ni-dependent
										11	Y	Y		
lpxT	-	N/S	-1.1[r]	0.6	GE GS ME	MicA	A	n.d.	n.d.	7	Y	Y		undecaprenyl pyrophosphate phosphatase
										11	Y	Y		
ompX[m]	-	S	-1.8	0.1	GE GS ME MS	MicA	B2	1-24	Y	7	Y	Y	Y/Y	outer membrane protein
										11	Y	Y		
ycfS	-	N/S	-2.3	-0.1	GE GS ME MS	MicA	B2	footnote[v]	n.d.	7	n.d	Y		L,D-transpeptidase; covalent attachment of peptidoglycan to the outer membrane
										11	n.d	N		
pal	none	S	-1.3	0.2	GE GS ME	MicA	C	n.d.	n.d.	7	n.d.	N		peptidoglycan-associated outer membrane lipoprotein
										11	n.d.	Y		
phoP	none	S	-1.0	-0.2	GE GS ME MS	MicA	B2	8-24	N	7	Y	Y	footnote[i]	DNA-binding response regulator in two-component regulatory system with PhoQ
										11	Y	Y		
ybgF	none	S	-1.1	-0.3	GE GS ME MS	MicA	A	n.d.	n.d.	7	n.d.	Y		ybgF predicted periplasmic protein member for Tol-Pal system
										11	n.d.	Y		
C. Targets of RybB														
fadL	-	N/S	0.3	-2.1	GE GS ME MS	RybB	B2	1-16	Y	2	Y	Y		long-chain fatty acid outer membrane transporter
fiu[j]	-	N/S	-0.5	-1.5	GE GS ME MS	RybB	A	1-16	Y	2	Y	Y	Y/Y	predicted iron outer membrane transporter
fumC	none	S	0.0	-1.3	GE	RybB	B2	n.d.	n.d.	2	n.d	n.d.		fumarate hydratase, aerobic Class II
hinT	none	S	0.2	-1.1	GE GS ME MS	RybB	B2	1-16	Y	2	Y	Y		purine nucleoside phosphoramidase
nmpC	-	S	-0.5	-1.7	GE ME MS	RybB	B2	1-16	Y	2	Y	Y	Y/Y	OM porin; locus of qsr prophage, silent gene in *E.coli* K-12, homologous to ompD in *Salmonella*
ompF[m]	-	S	-0.4	-3.4	GE GS ME MS	RybB	A	1-16	Y	2	Y	Y	Y/Y	outer membrane porin 1a
ompC	-	N/S	0.3	-3.3	GE GS ME MS	RybB	A	1-16	Y	2	Y	Y	Y/Y	outer membrane porin protein C
rluD	-	N/S	0.1	-1.2	GE ME MS	RybB	B2	1-16	Y	2	Y	Y	Y/Y	23S rRNA pseudouridine synthase
asr	none	S	0.4	-2.5	GE	RybB	B2	n.d.	n.d.	2	n.d	n.d.		acid shock-inducible periplasmic protein
fimA	none	S	0.3	-1.8	GE GS ME MS	RybB	B2	1-16	Y	2	N	N		major type 1 subunit fimbrin (pilin)
rbsK	none	S	0.0	-1.9[q]	GE GS ME MS	RybB	A	n.d.	n.d.	2	n.d.	n.d.		ribokinase
rbsB	none	S	-0.1	-1.5[q]	GE ME MS	RybB	A	1-16	Y	2	Y	Y		D-ribose transporter subunit
rraB	none	S	0.1	-1.1[r]	GE GS ME MS	RybB	B2	full length[w]	Y	2	Y	Y		ribonuclease E inhibitor protein
ycfL	none	S	0.0	-1.1	GE GS ME MS	RybB	B1	1-16	Y	2	Y	Y		ycfL predicted protein
ydeN	none	S	0.3	-1.2[q]	ME MS	RybB	B2	full length[w]	Y	2	N	N		conserved protein, puative sulfatase
yhjJ	+	U	-0.2	-1.3[r]	GS MS	RybB	B2	1-16	n.d.	2	n.d	n.d.		predicted zinc-dependent peptidase

[a] Effect on target gene mRNA levels after σ^E overexpression as measured by qRT-PCR. **-** and **+** denotes target down- or up-regulated ≥2-fold in at least one condition, respectively; **none** denotes no change.

[b] Requirement of sRNA(s) for target gene regulation. **N**, sRNA(s) necessary, with ≤2-fold decrease in target level as determined by σ^E overexpression in a MicA and/or RybB deletion strain: measured by qRT-PCR in at least one condition. **S**, sRNA(s) sufficient, with ≥2-fold decrease in target level after MicA or RybB overexpression measured by microarray in at least one condition, and ≥ 1.5 fold in additional conditions. **U**, unique sRNA(s) regulation, as σ^E overexpression in MicA and/or RybB deletion strain gives ≥2 fold upregulation but MicA or RybB overexpression gives ≥2 fold downregulation.

[c] Observed log2 fold change in target gene mRNA levels compared to wild type as measured by microarray after overexpression of either MicA or RybB during exponential growth in minimal media with glucose, unless otherwise indicated.

[d] Growth conditions with significant regulation (≥2-fold) of target gene mRNA after sRNA overexpression as determined by microarray. **GE** Glucose Exponential; **GS** Glucose Stationary; **ME** Maltose Exponential; **MS** Maltose Stationary.

[e] Region of sRNA interaction on target mRNA as predicted by RNAhybrid. Nucleotide positions relative to start codon. **A** is 5' to the upstream boundary of the ribosome footprint (≥ - 21 nt) **B** is within the ribosome footprint (**B1** -20 to -12; **B2** -11 to +3; **B3** +4 to +15), with **B2** overlapping the SD and AUG start codon; **C** is downstream (3') of the ribosome footprint (≥+16 nt)

[f] Observed minimal 5' region of sRNA sufficient to downregulate the target mRNA ≥2-fold.

[g] Predicted effect of minimal sRNA truncation on target mRNA levels; **Y** predicted to regulate; **N** predicted not to regulate

[h] Observed and predicted effects of overexpression of mutant sRNAs on target gene mRNA levels. Position: location of C to G point mutation; Abrogate: observed effect with **Y** indicating abrogation and **N** indicating no effect on target mRNA levels; Predicted: denotes whether nucleotide change is predicted **Y** or not predicted **N** to affect mRNA levels

[i] Effect of compensatory mutation on mRNA for mutant sRNA. **Y/Y** indicates that the compensatory mutation was tested and restored downregulation of the target mRNA, **Y/N** indicates it was tested but did not restore downregulation.

[j] ≥2-fold downregulation seen in GE condition for both MicA and RybB, due to ability of the microarray to detect low levels of *lamB*, as *lamB* is in low abundance when grown with glucose.

[k] N/S in ME, S for GE

[l] Additional regulation observed in GS that is dependent on MicA.

[m] Additional regulation observed during σ^E overexpression in the ΔsRNA strain.

[o] Necessity not tested in ME MS

[p] GE condition for MicA is not a ≥2-fold decrease in target

[q] data from ME

[r] data from GS

[s] data from MS

[t] shown previously in *E.coli*

[u] tsxM2, which is specifically mutated only in the Ryb-Tsx binding region, is unable to be regulated by either MicA or RybB, suggesting altered secondary structure that occludes regulation.

[v] *ycsF* mRNA is regulated by the omrB fusion alone, so this method does not allow for examination of regulation by MicA.

[W] For *ydeN*, RybB (1–16nt) was predicted not to be sufficient for downregulation, as observed. For *rraB*, RybB (1–16nt) was predicted to downregulate, but downregulation was not observed.

Table S2 Log2 fold change values of mRNA as measred by Microarray or qRT-PCR

		Microarray data									qRT-PCR data														
UNIQID	NAME	G.E. pMicA	G.S. pMicA	M.E. pMicA	M.S. pMicA	G.E. pRybB	G.S. pRybB	M.E. pRybB	M.S. pRybB	G.E. pRpoE	G.E. pRybB	G.E. pMicA	G.E. drybB pRpoE	G.E. dmicA pRpoE	G.E. dmicA drybB pRpoE	G.S. pRpoE	M.E. pRpoE	M.E. pRybB	M.E. pMicA	M.E. drybB pRpoE	M.E. dmicA pRpoE	M.E. dmicA drybB pRpoE	M.S. pRpoE	M.S. pRybB
b4036	lamB	-1.53	-0.11	-1.87	-1.50	-2.41	-0.30	-2.08	-1.52								-2.03	-2.36	-2.21	-1.42	-1.87	-0.08		
b0957	ompA	-2.60	-2.58	-2.44	-2.72	-1.16	-1.49	-1.16	-1.83	-2.97	-1.08	-3.16	-2.45	-0.70	-0.14									
b1256	ompW	-0.57	-0.11	-1.09	-0.29	-1.91	-0.43	-2.21	-0.42	-1.41			-0.23	-0.87	0.08		-1.33			-1.21	-0.84	-0.94		
b0411	tsx	-2.30	-1.39	-2.38	-1.15	-3.19	-1.89	-3.10	-2.85	-2.22	-2.44	-2.16	-1.47	-1.60	-0.21									
b3055	htrG	-0.51	-1.10	-0.78	-0.61	-0.82	-2.05	-1.13	-1.73							1.98	4.50			4.07	4.93	4.26	2.60	
b2419	yfeK	-0.92	-0.85	-0.84	-0.79	-0.80	-1.72	-1.63	-1.67							2.98	6.59	-2.12	-1.44	6.23	6.37	6.25	3.30	
b4411	ecnB	-0.58	-1.23	-0.56	-2.67	0.26	1.09	0.61	0.46	-0.54			-0.28	-0.03	-0.21	-0.41							-1.03	
b4312	flmB	-1.55	-1.06	-0.94	-0.66	0.17	-0.09	0.31	0.21	-1.47	0.36		-0.93	-0.54		-0.21								
b1651	gloA	-1.45	-1.23	-1.35	-2.12	-0.05	0.43	0.38	0.20	-0.26			-0.54	-0.88	0.01		-0.01			-0.62	0.21			
b2174	lpxT	-0.96	-1.09	-1.06	-0.26	0.60	-0.63	-0.02	-0.47	-1.06			-0.66	-0.10	-0.06		0.04			-0.56	-0.42			
b0814	ompX	-1.80	-1.85	-1.17	-1.92	0.13	0.78	0.93	0.43	-2.99	0.18	-2.00	-2.68	-0.98	-0.91									
b1113	ycfS	-2.34	-2.01	-2.51	-1.95	-0.12	-0.06	-0.19	0.32	-2.08				-0.16		-0.73	-1.58			-2.03	0.35			
b0741	pal	-1.29	-1.37	-1.33	-0.44	0.17	0.17	0.76	-0.11	-0.72				0.61		0.07	-0.03			0.34	0.82	0.96		
b1130	phoP	-1.05	-0.86	-0.85	-0.70	-0.18	0.15	0.60	0.38	-0.58				-0.03		0.05								
b0742	ybgF	-1.07	-0.93	-1.20	-0.64	-0.29	-0.34	-0.02	-0.13								0.26			0.43	0.81	0.87		
b2344	tktL	0.32	-0.30	-0.06	0.41	-2.09	-2.03	-2.93	-1.51	-1.26	-1.70	-0.03	0.07	-1.77	-0.09									
b0805	flu	-0.46	-1.50	1.14	-0.23	-1.50	-2.34	-2.05	-2.88	-1.00	-1.65	0.45	0.09	-1.59	0.06									
b1611	fumC	0.02	0.57	-0.08	0.56	-1.27	1.97	-0.49	-0.61	-0.68			-0.10	-0.71	-0.31									
b1103	hinT	0.21	0.38	0.10	0.09	-1.05	-0.89	-0.72	-0.92	-0.81	-0.78		-0.25			-0.13							-0.42	-0.98
b0553	nmpC	-0.50	0.17	0.25	0.69	-1.68	-0.16	-1.52	-1.08	-4.00	-1.26	-0.21	-3.09	-3.62	-2.12									
b0929	ompF	-0.39	-0.06	0.73	0.75	-3.37	-2.35	-2.57	-1.79	-5.46	-3.40	0.01	-1.44	-5.50	-1.27									
b2215	ompC	0.31	1.10	0.43	1.28	-3.34	-2.30	-2.62	-2.33	-4.12	-3.33	0.22	-0.23	-4.18	-0.12									
b2594	rluD	0.07	-0.32	-0.52	0.14	-1.19	-0.33	-1.24	-0.67	-1.01	-0.85		-0.68											
b1597	asr	0.45	0.70	-0.02	-0.52	-2.52	0.53	0.21	0.11	-0.23	-3.11		-3.38	0.05		0.83								
b4314	flmA	0.28	0.20	0.19	0.09	-1.79	-2.87	-2.39	-2.44	-0.12	-1.40		0.67											
b3752	rbsK	-0.05	0.26	-0.42	0.30	-0.85	-0.76	-1.87	-1.40								0.60			0.69	0.62			
b3751	rbsB	-0.13	1.07	-0.35	0.33	-0.87	0.10	-1.46	-0.96								0.65			0.99	0.59			
b4255	malB	0.10	0.16	-0.17	-0.03	-0.94	-1.15	-0.72	-1.13	-0.42	-1.29		-0.40			0.18							-0.28	-0.79
b1104	ycfL	0.00	0.44	-0.11	-0.05	-1.09	-1.25	-0.79	-0.98	-0.65			-0.03	-0.72	-0.23									
b1498	ydeN	0.32	-0.13	-0.22	-0.25	-0.07	-0.18	-1.18	-1.34								0.78			0.94	0.58		-0.29	-1.81
b3627	yhiJ	-0.20	-0.23	-0.13	-0.58	-0.03	-1.31	-0.58	-1.06							2.47							2.45	-1.28

Table S3 Strains and Plasmids Used in This Study

Common Name	Database #	Comment	Reference
MG1655	CAG45114	*E. coli* K-12 (MG1655) rph-1	*E.coli* Genetic Stock Center
delta *micA*	CAG62031	P1 transductant of CN 2810 (Molecular Microbiology 58 (5), 1421–1429) micA::cam into CAG45114.	this study
delta *rybB*	CAG62002	P1 transductant of KMT197 (J. Bacteriol. Thompson et al. 189: 4243) rybB::kan into CAG45114.	this study
delta *micA* delta *rybB*	CAG62097	P1 transductant of KMT197 lysate into CAG62031 Kan-Cam	this study
delta *cyaR*	CAG62212	P1 transducant of GSO145 (J. Bacteriol. Hobbs et al. 192:1) cyaR::kan into CAG45114.	this study
delta *ompA*	CAG62165	from the Keio collection, ompA::kan	Baba et al. 2006
pLC245	CAG25197	IPTG inducible plasmid with trc promoter driving *rpoE* expression	Rhodius et al. 2006
p*Trc*99a	CAG25196	IPTG inducible plasmid with trc promoter	Amersham Pharmacia Biotech
pXG-10	CAG62318	pXG-10 plasmid for making constituitively expressed, in frame translational fusions, of target mRNA to *gfp*.	Urban and Vogel 2007
pJV300	pJV300	control plasmid for *gfp* fusion assays	Sittka et al. 2007
ptrcEGoperator	CAG62157	derivative of ptrcEGoperator with an operator site betweeen the -10 and -35 to remain IPTG-inducible, with an MscI cloning site located adjacent to the promoter to clone sRNAs without adding additional sequence to the transcript.	this study
pRpoE	CAG25197	MG1655 with pLC245	Rhodius et al. 2006
pRybB	CAG62150	*rybB* cloned in ptrcEGoperator downstream of the IPTG inducible trc promoter	this study
pMicA	CAG62156	*micA* cloned in ptrcEGoperator downstream of the IPTG inducible trc promoter	this study
delta *rybB* pRpoE	CAG62122	CAG25197 transformed into CAG62002	this study
delta *micA* pRpoE	CAG62130	pLC245 transformed into CAG62031	this study
delta *micA* delta *rybB* pRpoE	CAG62132	pLC245 transformed into CAG62097	this study
delta *cyaR* pRpoE	CAG62220	pLC245 transformed into CAG62212	this study
delta *micA* delta *cyaR* pRpoE	CAG62214	P1 transductant of CAG62212 into CAG62130	this study
fullmicA	CAG62275	full length MicA cloned into CAG62289, creating a micA-omrB fusion.	this study
micA 1-57	CAG62277	MicA missing the last 15nt from the 3' end, cloned into CAG62289, creating a micA 1-57-omrB fusion.	this study
micA 1-24	CAG62282	The first 24 nt of MicA, cloned into CAG62289, creating a micA 1-24-omrB fusion.	this study
micA 8-24	CAG62284	The 8-24 nt of MicA, cloned into CAG62289, creating a micA 8-24-omrB fusion.	this study
full rybB	CAG62285	full length RybB cloned into CAG62289, creating a micA-omrB fusion.	this study
rybB 1-16	CAG62287	The first 16nt of RybB cloned into CAG62289, creating a rybB 1-16-omrB fusion.	this study
omrB	CAG62289	nucleotides 16-85 of omrB cloned into CAG62157 with a 5' XbaI site for creating sRNA-omrB fusion constructs.	this study
micAM7	CAG62307	MicA with a C to G change at position 7, cloned into CAG62289, creating a micAM7-omrB fusion.	this study
micAM11	CAG62309	MicA with a C to G change at position 11, cloned into CAG62289, creating a micAM11-omrB fusion.	this study
micAM7M11	CAG62311	MicA with a C to G change at position 7, and a C to G change at position 11, cloned into CAG62289, creating a micAM7M11-omrB fusion.	this study
rybBM2	CAG62290	RybB with a C to G change at position 2, cloned into CAG62289, creating a rybBM2-omrB fusion.	this study
pRseAB; '+σ^E shutoff"	CAG62691	referred to as '+σ^E shutoff' plasmid in text; is an IPTG inducible plasmid with trc promoter driving *rseAB* expression	Costanzo and Ades 2008
pXG-RybB; 'RybB'	CAG62692	referred to as +sRNA plasmid 'RybB' in text; is *rybB* with IPTG inducible trc promoter cloned into pXG-10 with *gfp* removed	this study
pXG-MicA; 'MicA'	CAG62693	referred to as +sRNA plasmid 'MicA' in text; *micA* with IPTG inducible trc promoter cloned into pXG-10 with *gfp* removed	this study
+σ^E shutoff", '- sRNA'	CAG62689	CAG62691 and CAG62318	
+σ^E shutoff", 'RybB'	CAG62683	CAG62691 and CAG62692	
+σ^E shutoff", 'MicA'	CAG62684	CAG62691 and CAG62693	
- σ^E shutoff", '- sRNA'	CAG62697	CAG25196 and CAG62318	
-σ^E shutoff", 'RybB'	CAG62692	CAG25196 and CAG62692	
-σ^E shutoff", 'MicA'	CAG62693	CAG25196 and CAG62693	
pfiu::gfp	pKP-192-1	pXG-10 backbone with target as indicated in common name	this study
prluD::gfp	pKP-210-1	pXG-10 backbone with target as indicated in common name	this study
pfiu::gfp*	pKP-209-1	pXG-10 backbone with target as indicated in common name	this study
prluD::gfp*	pKP-218-3	pXG-10 backbone with target as indicated in common name	this study
pFM-1-1/ WT rybB	CAG62224	pJV300 backbone with sRNA as indicated in common name	Bouvier et al. 2008
pFM-17-2/ rybBM2/ RybB*	CAG62290	pJV300 backbone with sRNA as indicated in common name	Bouvier et al. 2008
WT rybB, WT ompA	CAG62582	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, ompAM2	CAG62587	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, ompAM2	CAG62590	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, WT ompA	CAG62579	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, ompAM2	CAG62591	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, WT ompC	CAG62375	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, ompCM2	CAG62503	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study

WT rybB, ompCM2	CAG62506	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, WT ompC	CAG62446	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, WT nmpC	CAG62350	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, nmpCM2	CAG62497	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, nmpCM2	CAG62500	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, WT nmpC	CAG62431	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, WT ompX	CAG62634	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micAM7, ompXM7	CAG62628	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, ompXM7	CAG62627	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micAM7, WT ompX	CAG62635	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, WT ompF	CAG62546	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, ompFM2	CAG62455	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, ompFM2	CAG62470	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, WT ompF	CAG62543	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, WT tsx	CAG62355	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micAM11, tsxM11	CAG62493	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, tsxm11	CAG62495	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micM11, WT tsx	CAG62427	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, tsxM11	CAG62494	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, lamBM11	CAG62555	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micAM11, WT lamB	CAG62430	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, lamBm11	CAG62556	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
micAM11, lamBm11	CAG62554	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, WT lamB	CAG62356	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, WT tsx	CAG62348	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBm2, tsxM2	CAG62464	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT rybB, tsxM2	CAG62479	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
rybBM2, WT tsx	CAG62425	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
WT micA, tsxM2	CAG62480	pJV300 backbone with sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, nmpC WT	CAG62601	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, ompF WT	CAG62502	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB-, ompC WT	CAG62472	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, nmpC M2	CAG62403	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, ompF M2	CAG62597	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB-, ompC M2	CAG62626	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
MicA -, ompX WT	CAG62584	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
MicA -, ompX M7	CAG62592	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, ompA WT	CAG62323	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, ompAM2	CAG62496	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
MicA -, tsx WT	CAG62607	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
MicA -, tsxM11	CAG62481	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB -, tsxWT	CAG62607	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study
RybB-, tsxM2	CAG62481	pJV300 backbone with no sRNA, pXG-10 backbone with target as indicated in common name	this study

Table S4 Primers, probes and oligonucleotides used in this study

Primer Name	Sequence	Use	Comment
ptrc_EGtop	\5Phos\ATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGGCCATACAGAC	plasmid construction	For adding operator site between the -10 and -35 of CAG25196, also adds MscI site for cloning sRNAs without additional plasmid sequence in the transcript
ptrc_EGbottom	\5Phos\CATGGTCTGTATGGCCATTATACGAGCCGGATGATTAATTGTCAACAGCTCATTTCAGAAT	plasmid construction	For adding operator site between the -10 and -35 of CAG25196, also adds MscI site for cloning sRNAs without additional plasmid sequence in the transcript
R_fullmicA_XbaI	TGACTCTAGAAAAAGGCCACTCGTGAGTGG	plasmid construction	For adding XbaI site to 3' end of full length MicA
L_omrBsalm_XbaI	TGACTCTAGAAGGTGGAATCAACGTCATTGTT	plasmid construction	For adding XbaI site to 5' end of OmrB
HindIII_omrB_R	TGACAAGCTTAGGTTCAGGCGAAACAAAAA	plasmid construction	For adding HindII site to 3' end of OmrB
R_omrBsalm	CGTTCACCGACAAACAACAG	plasmid construction	For amplifying omrB
R_micA#1_XbaI	TGACTCTAGAGATGATAACAAATGCGCGTCTTT	plasmid construction	For adding XbaI site to 3' end of MicA 1-24nt.
L_micA#2_MscI	CCATGCGCATTTGTTATCATCAGGTGGAATCAACGTCATTGTT	plasmid construction	For adding MscI to 5' end of MicA 8-24nt
R_micA#3_XbaI	TGACTCTAGAAGTGGCCAAAATTTCATCTCTGAA	plasmid construction	For adding XbaI on 3' end of MicA 3' -15nt
R_fullrybB_XbaI	TGACTCTAGAACAAAAAACCCATCAACGTTGAAC	plasmid construction	For adding XbaI site to 3' end of full length RybB
R_1_16rybB_XbaI	TGACTCTAGAAAAGAAAAGCAGTGGCATGGC	plasmid construction	For adding XbaI site to 3' end of RybB 1-16
MscI_micAconstruct2_L	CCATGCGCATTTGTTATCATC	plasmid construction	For cloning CAG62255 into CAG62157
L_rybBmscI_M2	CCATGGCACTGCTTTTCTTT	plasmid construction	For cloning the 5' end of RybB with the 2nt changed from a C to a G.
JVO-4796	gtttttgctagcTAAGCGTTGACCGAGTTG	GFP reporter assay	For cloning WT rluD into pXG-10
JVO-4798	gtttttttATGCATCAAAATTATTATCACTTTCACGAG	GFP reporter assay	For cloning WT fiu into pXG-10
JVO-4799	gtttttgctagcACCGGCAAAGAACGTGA	GFP reporter assay	For cloning WT fiu into pXG-10
JVO-5301	5'P~TAACAGTGCCATCGCATCC	GFP reporter assay	For cloning fiu with single nucleotide change into pXG-10
JVO-5302	ATCCCGTGATAGTCGTCGT	GFP reporter assay	For cloning fiu with single nucleotide change into pXG-10
JVO-5557	gtttttatgcatTATATTAAACGGCAAAGCC	GFP reporter assay	For cloning WT rluD into pXG-10
JVO-5656	tatgCcacaacgagtacagc	GFP reporter assay	For cloning rluD with singlenucleotide change into pXG-10
JVO-5657	TTGTGGCATATATACTGCTTCT	GFP reporter assay	For cloning rluD with singlenucleotide change into pXG-10
L_micA_cMscI	TGGCCATGAAAGACGCGCATTT	GFP reporter assay	For cloning WT full length micA into pJV300
L_micAM7_cMscI	TGGCCATGAAAGA**G**GCGCATTT	GFP reporter assay	For cloning full length micA with a C to G nucleotide change at the 7th nucleotide into pJV300
L_micAM11_cMscI	TGGCCATGAAAGA**G**GCG**G**ATTT	GFP reporter assay	For cloning full length micA with a C to G nucleotide change at the 11th nucleotide into pJV300
L_nsiI_ompC	tgacATGCATaaaagttttagtatcatattcgtgttg	GFP reporter assay	For cloning WT ompC into pXG-10
L_nsiI_ompF	tgacATGCATagacacataaagacaccaaactctca	GFP reporter assay	For cloning WT ompF into pXG-10
L_nsiI_ompX	tgacGCTAGCaagacatgcaatttttttcataaccac	GFP reporter assay	For cloning WT ompX into pXG-10
L_nsiI_tsx	tgacATGCATatcacctggattgatagtaaaagtttg	GFP reporter assay	For cloning WT tsx into pXG-10
L_nsiI_ompAv1	tgacATGCATacatgccaggggtgctc	GFP reporter assay	For cloning WT ompA into pXG-10
L_nsiI_nmpC	tgacATGCATtgaatatttatcgcgcagtga	GFP reporter assay	For cloning WT nmpC into pXG-10
R_NheI_ompC	tgacGCTAGCtgaaagacatgcaatttttttcataac	GFP reporter assay	For cloning WT ompC into pXG-10
R_NheI_ompF	tgacGCTAGCcagaatattgcgcttcatcattattta	GFP reporter assay	For cloning WT ompF into pXG-10
R_NheI_ompX	tgacGCTAGCagacatgcaatttttttcataaccac	GFP reporter assay	For cloning WT ompX into pXG-10
R_NheI_tsx	tgacGCTAGCtgccagtaatgtttttttcatatgtatg	GFP reporter assay	For cloning WT tsx into pXG-10
R_NheI_ompA	tgacGCTAGCagcgaaaccagccagtgc	GFP reporter assay	For cloning WT ompA into pXG-10
R_NheI_nmpC	tgacGCTAGCtgatgcagctacagcagaaa	GFP reporter assay	For cloning WT nmpC into pXG-10
nmpCM2	cgatgaaaaattaacagtgccaatttctgctgtagctgca	GFP reporter assay	For cloning nmpCM2 into pXG-10
nmpCM2_anti	tgcagctacagcagaaattggcactgttaatttttcatcg	GFP reporter assay	For cloning nmpCM2_anti into pXG-10
tsxM2_rybB	cgcaagggattttcaaacagtgccatacatatgaaaaaaacattac	GFP reporter assay	For cloning tsxM2_rybB into pXG-10
tsxM2_rybBanti	gtaatgttttttcatatgtatgcactgtttgaaaatcccttgcg	GFP reporter assay	For cloning tsxM2_rybBanti into pXG-10
tsxM11_micA	tgtatacgaaatatttagaaaccgcaatttccgccttttcactcc	GFP reporter assay	For cloning tsxM11_micA into pXG-10
tsxM11_micAanti	ggagtgaaaaaggccggaaattgcgtttctaaatatttcgtataca	GFP reporter assay	For cloning tsxM11_micAanti into pXG-10
ompFM2	gaacttattgacggcagtgccaggtgtcataaaaaaaacc	GFP reporter assay	For cloning ompFM2 into pXG-10
ompFM2_anti	ggttttttttatgacacctggcactgccgtcaataagttc	GFP reporter assay	For cloning ompFM2_anti into pXG-10
ompCM2	aggqttaatcagtatgcagtgccataaaaaaagcaaataaggc	GFP reporter assay	For cloning ompCM2 into pXG-10
ompCM2_anti	gcctttatttgctttttatggcactgcatactgattaaccct	GFP reporter assay	For cloning ompCM2_anti into pXG-10
ompAM2_rybB	gagcagatccccgctgaaggatttaaccg	GFP reporter assay	For cloning ompAM2_rybB into pXG-10
ompAM2_rybBanti	cggttaaatccttcagcgggggatctgctc	GFP reporter assay	For cloning ompAM2_rybBanti into pXG-10
ompXM7	aggtggttatgaaaaaaattgcatctcttttcagcactggc	GFP reporter assay	For cloning ompXM7 into pXG-10
ompXM7 antisense	gccagtgctgaaagaagatgcaatttttttcataaccacct	GFP reporter assay	For cloning ompXM7 antisense into pXG-10
rt_gyrA_L	GTCATGCCAACCAAAATTCC	qRT-PCR	control primer
rt_gyrA_R	ATGTGTTCCATCAGCCCTTC	qRT-PCR	control primer
rt_recA_L2	TCTACCGGTTCGCTTTCACT	qRT-PCR	control primer
rt_recA_R2	GCGTGTTCAGCATCGATAAA	qRT-PCR	control primer
rt_lpp L	GCTCCAGCAACGCTAAAATC	qRT-PCR	
rt_lpp R	CACGAGCTGCGTCATCTTTA	qRT-PCR	
rt_OmpF L	AGGCTTTGGTATCGTTGGTG	qRT-PCR	
rt_OmpF R	TGTTGTTCGCGTACTTC	qRT-PCR	
rt_yhcN L	CTGCACAAGCACAAAATCGT	qRT-PCR	
rt_yhcN R	GTAATCTGGTAGGCCGTTGC	qRT-PCR	
rt_OmpC L	CTACATGCGTCTTGGCTTCA	qRT-PCR	
rt_OmpC R	AATTTCAGACCTGCGAATGC	qRT-PCR	
rt_tsx L	TAGGGGATGACAGCGGTAAC	qRT-PCR	
rt_tsx R	TCAGTTCTGCATCGTCGTTC	qRT-PCR	
rt_OmpA L	TGAGTACGCGATCACTCCTG	qRT-PCR	
rt_OmpA R	TGACCGAAACGGTAGGAAAC	qRT-PCR	
rt_fiu L	CTCACCAACCAGACCAACCT	qRT-PCR	
rt_fiu R	GAATGCTGCTGTCAGGATGA	qRT-PCR	
rt_ompX_L	CAGGTACTTCCGTAGCTGC	qRT-PCR	
rt_ompX_R	CGGGTATTCAGTGGTCTGG	qRT-PCR	
sigmaE_RT_L1	GTCGTCCACCTTCCAGTGAT	qRT-PCR	
sigmaE_RT_R1	TAAATCTTCCGGGAGGGACT	qRT-PCR	
rt_lamB_L2	CTGAAGCTGGTGGTTCTTCC	qRT-PCR	
rt_lamB_R2	GACCGTAGTCGACACCCAGT	qRT-PCR	
rt_nmpC_L2	AGGTGAAACCCAAATCAACG	qRT-PCR	
rt_nmpC_R	CGTTGATTTGGGTTTCACCT	qRT-PCR	
yhjJ L	TACAGTGGCAAGTGCTGACC	qRT-PCR	
yhjJ R	TTAGCGCAATACGAGGGATG	qRT-PCR	
hinT L	TTCAGCAAAATTATTCGTCGTG	qRT-PCR	
hinT R	TGAGACGTCGTTCACAGTCG	qRT-PCR	
rluD L	TTCCCGGATTATTCACGTTC	qRT-PCR	
rluD R	GGATATCCTGCGGTTCAAAA	qRT-PCR	
rt_fimB_L	GGCGAGTGAAATTTGTCGAT	qRT-PCR	
rt_fimB_R	GCGGGTACGAAGTACGGATA	qRT-PCR	
rt_ecnB_L2	TGCAGCGATCTTTTCTGTTC	qRT-PCR	
rt_ecnB_R2	TTATTGCTGCGCTTTCGTT	qRT-PCR	
rt_fimA_L	GTTGATGCAGGCTCTGTTGA	qRT-PCR	
rt_fimA_R	AGCGGCTTTAGATGCAACAT	qRT-PCR	
phoP L	AGGATGCTGGTCATCAGGTC	qRT-PCR	
phoP R	ACCAGAATCGGCAGTGAAAC	qRT-PCR	
rt_yfeK_L2	CGAAGTTAACTGCCCATGAA	qRT-PCR	
yfeK R	GGTATTGCCGAGCTTCAGAC	qRT-PCR	
rt_gloA_L2	AAAGTGCTGGGCATGAAACT	qRT-PCR	
rt_gloA_R2	GCAGTGCCGAGTTCGTATT	qRT-PCR	
rt_ycfS_L2	CGTTTTTCTCGCTGGCTAAC	qRT-PCR	
rt_ycsf_R2	ACCGTCATTTTCCACCACAT	qRT-PCR	
fumC_L	TACTGGCAGGACAGCATGAC	qRT-PCR	
fumC_R	ACGTCGTCGTTAGGGTGAAC	qRT-PCR	
ompW_L7	TATTTTGGTGATGCCAGCAG	qRT-PCR	
ompW_R7	CTCCCCAGGAATCTTTCAGA	qRT-PCR	
htrG L3	CGCTATGTTTCCGACGAACT	qRT-PCR	
htrG R3	GGTGCTTGCGTCAGTTTGTA	qRT-PCR	
rt_fadL_L	CAGTCGCAGTGGCACTTATC	qRT-PCR	
rt_fadL_R	GTAATCAATGCGGGGTTACG	qRT-PCR	
rt_ompN_L	TTTTAATTCCTGCCCTGCTC	qRT-PCR	
rt_ompN_R	TAGCTCTGGTCGCCATCTTT	qRT-PCR	
N_micA	\5Biosg\GCCACTCGTGAGTGGCCAAAATTTCATCTCTGA	Probe	For Probing Northern Blots
N_RybB	\5Biosg\CAAAATGGGGACATCAAAGAAAAGCAGTGGC	Probe	For Probing Northern Blots
ompF_2_11	\5Biosg\CACCGTTACCCTTGGAAAAATAATGCAGACCAACAGCTTTACCG	Probe	For Probing Northern Blots
ompX_1_14	\5Biosg\AATGCGGTAAGCCGGACCAGCAGTGATGCC	Probe	For Probing Northern Blots
ompA_1_14	\5Biosg\CGATGGTGTGTGCGTCACCGATGTTGTTGG	Probe	For Probing Northern Blots

CHAPTER 2

Discovery and characterization of new sRNAs in the σ^E regulon

I. Background

Analysis of predicted σ^E dependent promoters (unpublished) suggested that there could be many more short transcripts spread throughout the genome that may encode for sRNAs. A key element of the σ^E response is the rapid degradation of a specific group of mRNAs (3). As these mRNAs decrease significantly within 10 minutes, and the decrease is dependent upon Hfq, it is likely that sRNAs may be involved in their regulation. To identify all σ^E or σ^{32} regulated sRNAs we sought an unbiased approach that could reveal sRNAs regardless of their target or mechanism of action. Given that sRNAs are typically between 50nt and 300nt in length (59, 60) and could be found anywhere in the genome, we utilized a tiling array platform (custom Affymetrix *E. coli* DNA tiling array) for sRNA discovery. This approach allows us to identify sRNAs with novel locations, such as those within the 3' or 5' of a protein encoding gene. We followed this analysis with confirmation of the putative sRNAs by 5' and 3' RACE and Northern analysis to reveal an approximate number of σ^E regulated sRNAs that may participate in the regulation of the outer membrane.

The cell is already known to employ small networks of sRNAs that are regulated by alternative sigma factors to achieve the necessary functionality for the cell. For example, the sigma factor σ^s employs three sRNAs devoted to the regulation of σ^s activity (1). As the central role of the σ^E response is to ensure that the protein components of the OM are folded and present in correct ratio to the LPS we hypothesized

that any new σ^E dependent sRNAs may also affect processes that are critical to the surveillance, maintenance and repair of cell envelope.

II. Results and Discussion

Identification of σ^E and σ^{32} regulated sRNAs

Previous work in our lab has determined that wildtype MG1655 *E.coli* grown in glucose minimal media during mid-exponential phase has a low basal level of σ^E activity and a reproducible transcriptome when profiled by DNA microarrays (4, 61). In addition, in a congenic strain carrying a σ^E expression plasmid driven by an IPTG inducible Ptrc promoter, overexpression of σ^E is sufficient to downregulate OMPs in as little as 20 minutes. We used this same overexpression strategy to identify σ^E regulated sRNAs. A strain carrying this σ^E overexpression plasmid was grown in glucose minimal media to mid-exponential phase, and a pre-induction sample (time 0) and a post-induction sample (time 20) was harvested. After hot phenol extraction of RNA each respective sample was hybridized to a custom Affymetrix *E. coli* tiling array, and an antibody specific for RNA-DNA complexes detected 'ON' tiles (in collaboration with lab of Dr. Gisela Storz). The resulting raw data set for each array was normalized and $\log_2$ transformed using the Tiling Analysis Software (TAS) to produce a 'normalized time 0 tiling data', and a 'normalized time 20 tiling data'. TAS was also be used to estimate the fold enrichment of tiles at time 20 compared to time 0, creating the 'fold enrichment at time 20 tiling data'. We then used the Integrated Genome Browser (IGB) to visualize these three data sets ('normalized time 0 tiling data', 'normalized time 20 tiling data' and 'fold enrichment at time 20 tiling data'), and to perform interval analysis with a 99.5% signal threshold to

determine significant features. Subsequently, the significant features were assessed according to their genomic location, presence of a predicted σ^E promoter, and predicted Rho-independent terminator (transtermHP). Additional software, Artemis, (62) was used to overlay σ^E promoter predictions (courtesy of V. Rhodius, unpublished data) with significant features. As a proof of principal my approach identified 100% of the σ^E regulon operons described previously (37/40 with a 99.5% threshold and the remaining 3 with a 98.5% threshold) and the two previously known σ^E sRNAs, MicA and RybB.

Next we sought to investigate whether these significant features encoded for new σ^E dependent sRNAs. First, we sought to experimentally determine their 5' and 3' ends and σ^E dependent promoter by using RACE. To determine the 5' and 3' ends of σ^E regulated candidates, RACE was performed (63) on samples from a strain lacking σ^E (Δ*rpoE*) and a σ^E overexpression strain. Subsequently, Northern analysis was performed as described previously (17) to confirm sRNA size, reveal any additional forms, and confirm regulation by σ^E (Figure 1, Figure 2). The summation of this analysis, compiled as a table of significant features classified as sRNAs, is shown in Table 1. While many significant tiling features were identified only those with additional experimental evidence (e.g. 5' RACE data) are included in Table 1.

Reg80 is differentially expressed in samples overexpressing either *rpoE* (σ^E) or *rpoH* (σ^{32}) by tiling array, however Northern analysis suggests a similar abundance of Reg80 whether or not *rpoH* was overexpressed. It is possible that the Reg80 signal may be at saturating levels (Figure 1) and is therefore not an accurate quantitation of the Reg80 signal during *rpoH* overexpression.

Interestingly, both Reg63 and Reg26 have at least two forms (Reg63: 280 nt, 180 nt and possibly 100nt; Reg26: 299 nt and 216 nt) (Figure 1, Figure 2C). This is not completely unexpected, as other sRNAs have been found with alternative forms (64, 65). However, it is novel to find sRNAs within protein encoding genes on the same strand. Curiously, both Reg63 and Reg26 are located within protein encoding genes and have alternative forms. Reg63 is located within the 5' of *yniA*, and Reg26 is within the 3' of *cutC* (Figure 1B, Figure 2A-B).

Next, we sought to fully characterize the novel sRNA Reg26. Analysis of the Reg26 promoter indicated that it has a high degree of sequence homology to the other known σ^E dependent sRNAs MicA and RybB (Figure 2D). Analysis of the Reg6 promoter strength *in vivo* found that the Reg26 promoter behaves almost identically to the other σ^E dependent sRNAs, MicA and RybB. This is in contrast to other members of the σ^E regulon that have lower promoter strengths (Figure 2E) (3). In addition, 5' and 3' RACE in the presence (σ^E +) or absence (σ^E -) σ^E confirms that Reg26 is dependent upon σ^E for transcription (Figure 2F). Taken together, this data demonstrates that Reg26 is a member of the σ^E regulon and adds further evidence to the hypothesis that σ^E gives primacy to the sRNA arm of the regulon (66).

Reg26 is an important member of the σ^E regulon

We sought to characterize the function of Reg26 by investigating its role in the σ^E response. Much like MicA and RybB, the abundance of Reg26 is dependent upon σ^E activity (Figure 3) (66). As many members of the σ^E regulon serve to monitor or repair the outermembrane we investigated whether Reg26 was required for of a variety of

growth conditions that cause perturbations to the outermembrane (Table 2; 17 different conditions). Wildtype and strains missing Reg26 (Δ*cutC*) were grown in regular LB overnight and plated on solid media to determine the colony morphology and plating efficiency for each condition. In addition, these strains were also assessed for their ability to grow in liquid culture by OD600.

When grown in liquid culture growth defects were seen at both 30°C and 37°C when are strains missing Reg26 (Δ*cutC*) (Figure 4A). This phenotype was fully complemented by pTrc99a driven expression of Reg26 from a plasmid (Δ*cutC* pReg26). This data suggest that Reg26 is important for maintaining proper growth in either slow (30°C) or fast (37°C) growth conditions.

Only one other condition, the addition of EDTA, yielded any significant Reg26-dependent phenotype. When grown in liquid culture at 37°C with 0.5mM EDTA present strains lacking Reg26 (Δ*cutC*) have a significant decrease in their OD600 when compared to a WT strain (WT). IPTG induced expression of Reg26 fully restores growth to that of WT, regardless of whether the expression is at a low level (0mM IPTG; pTrc99a is known to be slightly active in with no IPTG present), or maximally induced (1mM IPTG). This growth defect results in a 2-fold drop in the number of viable cells, even though the OD600 remains flat. Taken together this data indicate that EDTA exposure halts growth, although, it is not strongly bactericidal (Figure 4B). Thus, loss of Reg26 results in a bacteriostatic phenotype that is exacerbated with a particular stress to the outer membrane, the addition of EDTA. However, if cells are grown on solid media this phenotype is not exacerbated (Figure 5). Cells exhibit a small colony size regardless of whether EDTA is present (Figure 5A), and they do not have reduced cell viability when

plated (Figure 5B). Growth on solid media may provide an environment where the increase challenge of growth with EDTA is mitigated by other factors. The other factors may include the cell-cell contact afforded by growth as a colony, or a slower rate of division.

EDTA is known to remove stabilizing divalent cations from their binding sites in LPS that may result in the release of LPS from the outer membrane. When severe, this can lead to a rupture in the outer membrane (67). This is not dissimilar to the action of Na^+ or SDS on the membrane, and why under the conditions tested, Reg26 was not found to be necessary for normal growth under those conditions remains an open question.

Reg26 targets the lipoprotein Lpp

Earlier work had found that in response to overexpression of σ^E the mRNA of *lpp* decreased in abundance. Moreover, the *RNAhybrid* algorithm (42) predicts a a significant interaction between Reg26 and *lpp* (Figure 6A). Like the confirmed interactions of MicA and RybB with many of their targets Reg26 probably utilizes a GC rich sequence in its' 5' end to base pair with the *lpp* mRNA.

We tested whether Reg26 could target *lpp* mRNA for downregulation by assaying the abundance of *lpp* mRNA by Northern (Figure 6A), qRT-PCR (Figure 6B-C). Expression of Reg26 from a plasmid is sufficient to decrease the abundance of *lpp* mRNA (Figure 6A; WT pReg26 ,+IPTG). Reg26 is also necessary for this decrease, as strains missing Reg26 (Δ*cutC* pRpoE) do not show a significant decreases in *lpp* mRNA after overexpression of σ^E. Importantly, these effects are specifically mediated by Reg26 and are not due to the deletion in *cutC*. When just Reg26 is supplied on a plasmid it is

sufficient to restore downregulation of the *lpp* mRNA (Δ*cutC* pReg26). It is striking that Reg26 targets one of the most abundant proteins in the cell, the lipoprotein Lpp. Lpp is known to be necessary for the stabilization and integrity of the bacterial cell envelope by physically tethering the outer membrane to the peptidoglycan layer (68). As the σ^E response is an important surveillance and repair system for the cell envelope it is not surprising that it regulates Lpp. However, the advantages of using Reg26 in this way to downregulate the mRNA of *lpp* is unclear. The most direct consequence would be to stop the flow of Lpp to the perisplasm. However, cells without sufficient Lpp have a variety of defects in the outer membrane. Our data show that Reg26 acts to decrease the abundance of *lpp* mRNA, and that Reg26 is required for the cell to maintain normal growth. This suggests that the ability to lower levels of *lpp* mRNA plays a vital role in the cell, and that it is qualitatively different from experimental conditions where Lpp protein is depleted. We hypothesize that Reg26 is serving to alert an additional regulatory system to changes in the outer membrane, and thereby enacting that system to properly regulate the outer membrane. Reg26 initiates this additional regulatory system by decreasing the abundance of lpp *mRNA.* In the absence of this alert the cell must slow growth in order to compensate, as seen by the bacteriostatic phenotype of cells missing Reg26 (Figure 4) and their reduced colony size (Figure 5).

However, it is possible that like RybB and MicA, Reg26 has a variety of targets besides just *lpp*. To comprehensively define the target suite of Reg26 we used high-density tiling arrays to identify changes in mRNA abundance after short overexpression of Reg26 from an inducible plasmid. We used 4 different conditions to accommodate the possibility that regulation was growth-phase specific (exponential vs stationary phase) as

noted previously for the Hfq-associated ArcZ (38), or media specific (glucose vs maltose). However, this analysis only confirmed out previous findings that Reg26 is able to downregulate *lpp* mRNA, as no other transcripts were differentially regulated (data not shown). This suggests that the phenotypes we have observed in strains missing Reg26 are due to the lack of appropriate downregulation of *lpp* mRNA.

III. Figures

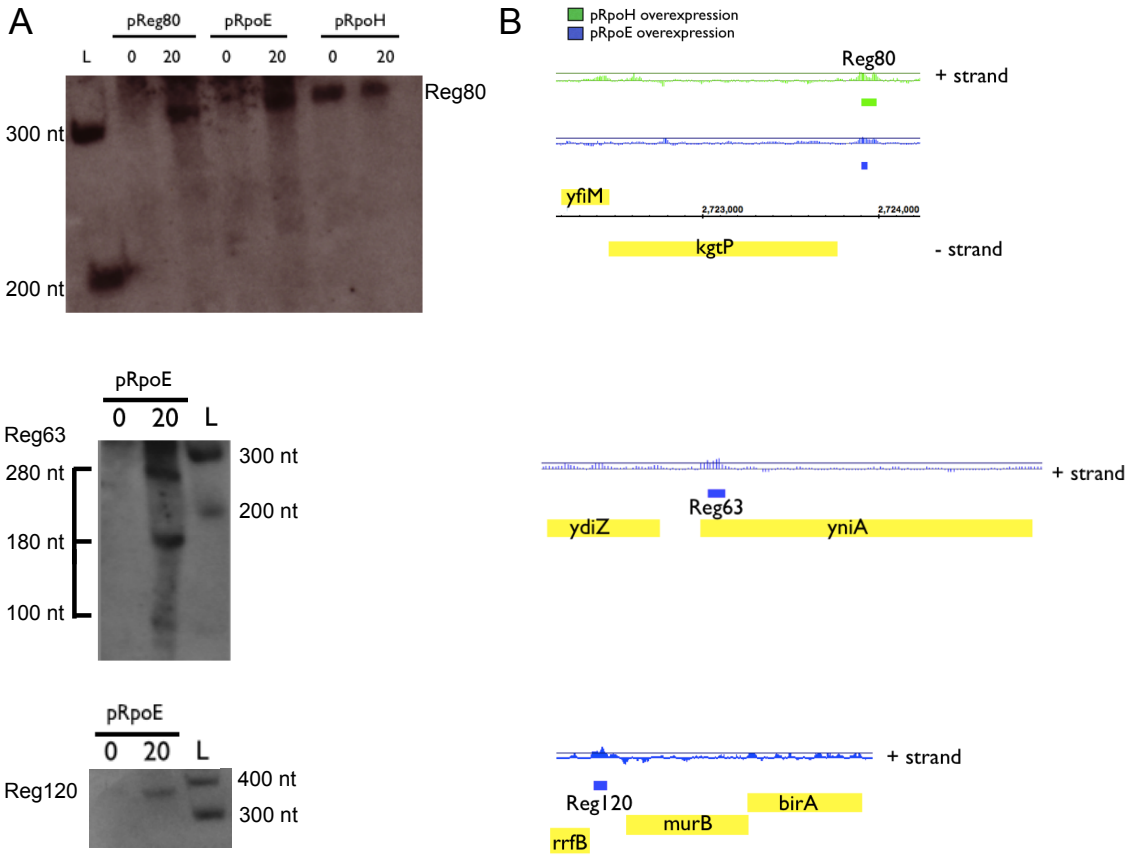

Figure 1. Identification of new σ^E and σ^{32} dependent sRNAs

Figure 1. Identification of new σ^E and σ^{32} dependent sRNAs

Examples of three new sRNAs. Northern Blots (A) and tiling array data (B) are shown. (A) Strains were grown overnight at 30°C in minimal media with glucose, diluted to OD450 = 0.03 in fresh minimal media with glucose and grown to OD450 = 0.3. At this point, a sample before induction ('No Induction') was harvested and the cultures were induced with 1mM IPTG. After 20 minutes a post-induction sample was harvested ('Induction'). RNA was prepared and the abundance of each sRNA assayed by Northern Blot. (B) Diagrams of each sRNA showing the tiling array data (green or blue trace; area of rectangle indicates a significant feature) overlaid with the genomic location (known protein encoding genes shown in yellow; DNA strand is given as + or -).

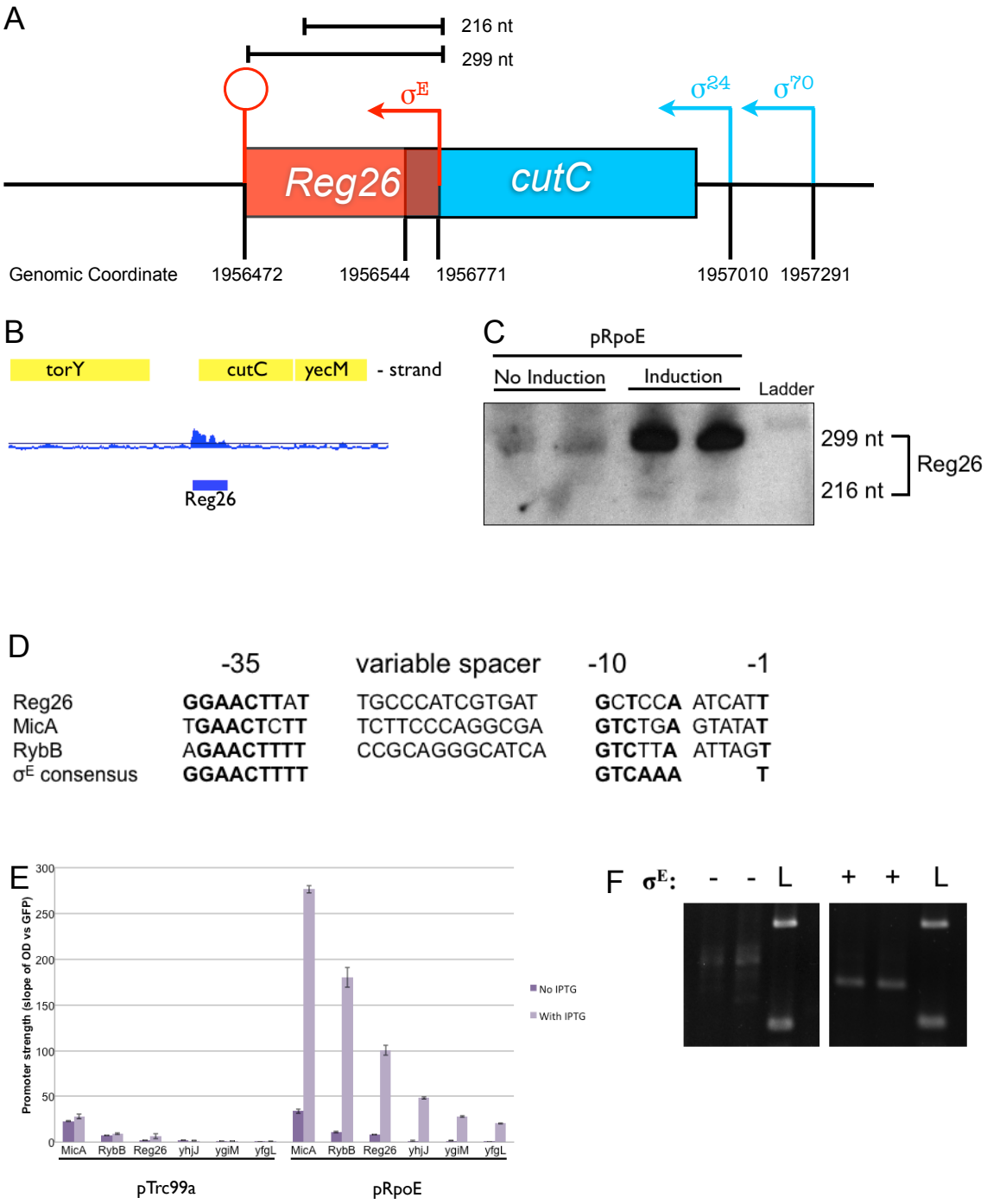

Figure 2. Reg26 is a σ^E dependent sRNA

Figure 2. Reg26 is a σ^E dependent sRNA

Reg26 has an RpoE dependent promoter that is similar in sequence and activity to the other known sRNAs with σ^E dependent promoters. Consequently, during expression of σ^E there is increased abundance of the 299nt (most abundant; corresponds to 5' mapped start through predicted terminator) and 216nt form (5' start to longest mapped 3' end) of Reg26. (A) Diagram of the sRNA Reg26 and the protein encoding gene *cutC*. The two different forms of the Reg26 sRNA are indicated as lines and their lengths (216nt and 299nt) given. (B) Diagram of tiling array data overlaid with genomic location as in Figure 1. (C) Strains were grown overnight at 30°C in minimal media with glucose, diluted to OD450 = 0.03 in fresh minimal media with glucose, and grown to OD450 = 0.3. At this point, a sample before induction ('No Induction') was harvested and the cultures were induced with 1mM IPTG. After 20 minutes a post-induction sample was harvested ('Induction'). RNA was prepared and the abundance of Reg26 assayed by Northern Blot. (D) Alignment of σ^E dependent sRNA promoters with the known σ^E consensus sequence. Conserved nucleotides are shown in bold. (E) Comparison of the promoter strengths of σ^E dependent transcripts without σ^E overexpression (empty plasmid; pUA66) a low level of σ^E overexpression (ptrc promoter driving expression of *rpoE;* pRpoE) and strong overexpression of σ^E (With IPTG, ptrc promoter driving expression of *rpoE;* pRpoE). Strains were grown overnight at 30°C in minimal media with glucose, diluted to OD450 = 0.03 in fresh minimal media with glucose and growth either with (+IPTG) or without (-IPTG) 100uM IPTG. Each strain contains two plasmids, one harboring the σ^E dependent promoter driving the expression of *gfp*; the second is the IPTG responsive promoter ptrc driving expression of *rpoE* or an empty vector. Promoter

strength is a function of the background-subtracted OD versus RFU differential rate plot for each reporter strain. The slope of the linear portion of each differential rate plot (OD600 0.25 to 0.55) corresponds to the promoter activity of the specific promoter-GFP fusion in that reporter strain. The average of three experimental values with the standard deviation shown is shown. (F) 5'-RACE experiments with Reg26-specific reverse primers designed to the 5' end of Reg26. L denotes the lane containing the ladder. The σ^E - indicates that cDNA was generated from a Δ*rpoE* strain; only PCR artifacts are present in these lanes. σ^E + indicates that cDNA was generated from a strain overexpressing σ^E; a single band is present that corresponds in length to the predicted σ^E dependent start site for Reg26. This band was sequenced and confirmed that it maps to the σ^E dependent start site for Reg26.

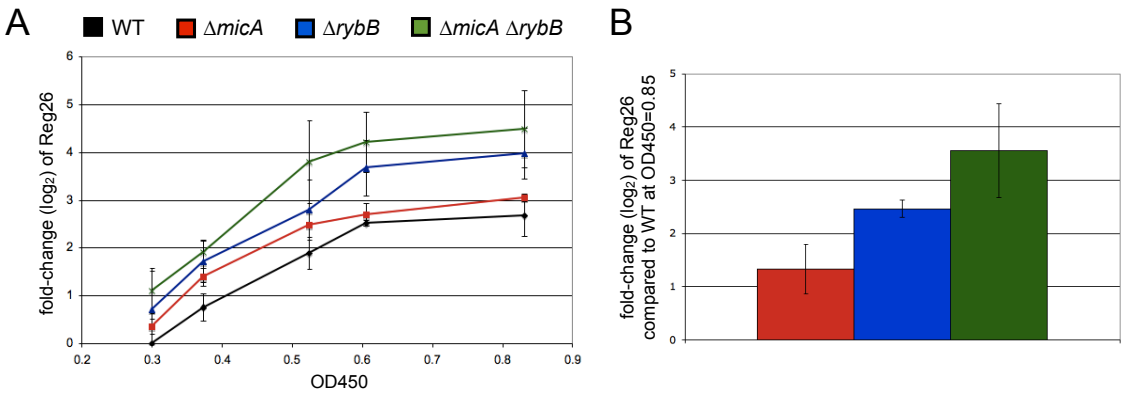

Figure 3. Reg26 abundance corresponds to the level of σ^E activity

Figure 3. Reg26 abundance corresponds to the level of σ^E activity

Panels A-B indicate the abundance of Reg26 in strains missing the σ^E dependent sRNAs MicA (Δ*micA*), RybB (Δ*rybB*) or both MicA and RybB (Δ*micA* Δ*rybB*). (A) The increased abundance of Reg26 corresponds to the previously known effects of these sRNAs on σ^E activity (66). (B) Shows the ratio of Reg26 transcript abundance compared to WT at OD450 ~ 0.8. For these experiments, strains were grown overnight at 30°C in minimal media with glucose, diluted to OD450 = 0.03 in fresh minimal media with glucose and samples harvested at the OD450 indicated. Samples were prepared for qRT-PCR and transcript abundance of each mRNA was quantified relative to WT at OD450 ~ 0.3 (A) or as indicated (B). The average of three experiments with standard deviations is shown.

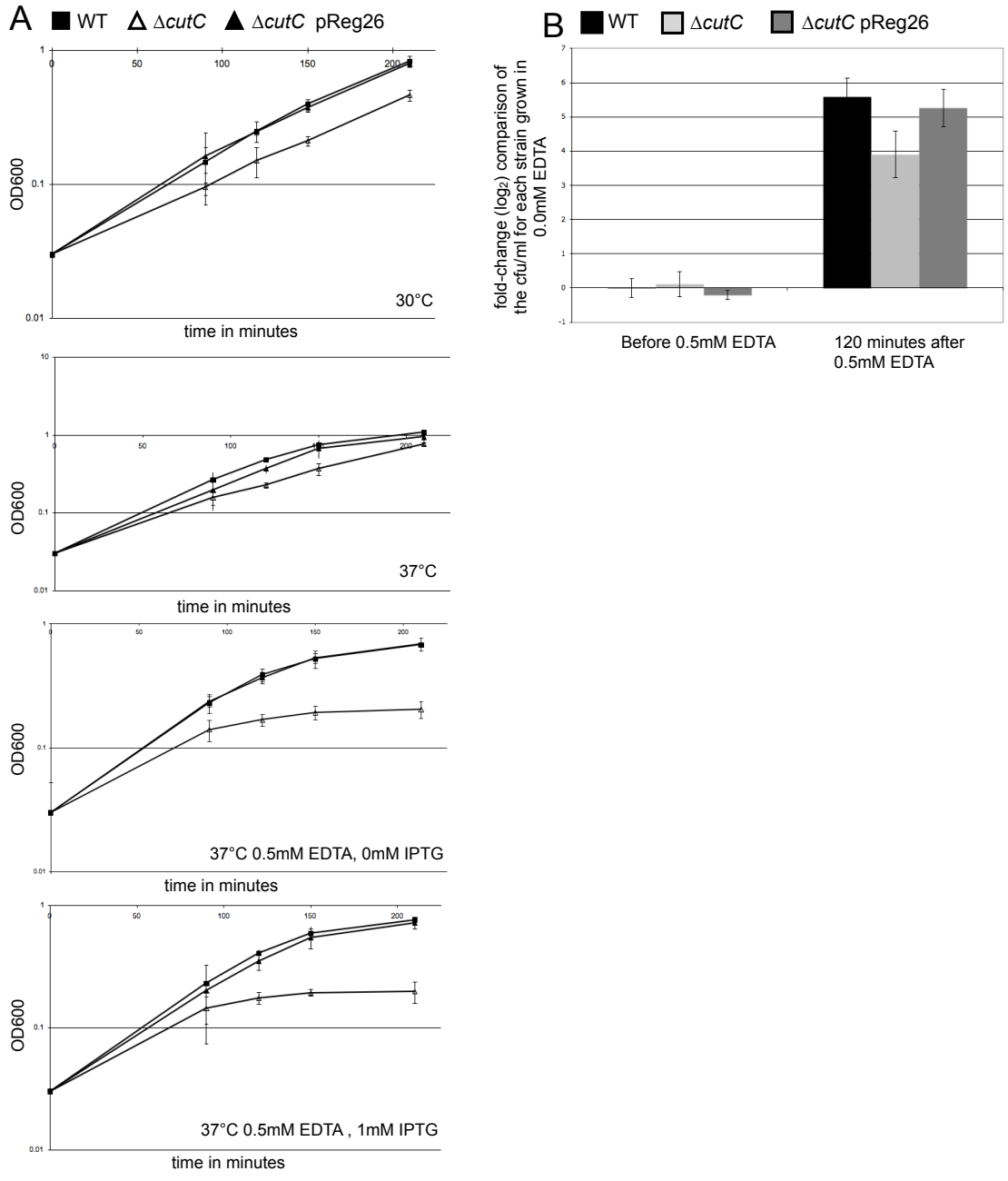

Figure 4. Loss of Reg26 results in a general growth defect that is exacerbated during liquid culture with EDTA

Figure 4. Loss of Reg26 results in a general growth defect that is exacerbated during liquid culture with EDTA

Panels A-B indicate that strains lacking Reg26 (Δ*cutC*) experience growth arrest in liquid media as indicated by the low OD600 values (A) and correspondingly low counts of viable cells as determined by cfu/ml (B).

(A) Strains lacking Reg26 (Δ*cutC*) exhibit lower OD600 readings throughout growth in liquid media when compared to WT strains grown under slow (30°C, LB) or fast (37°C, LB) growth conditions. This effect is most severe when EDTA is added at final concentration of 0.5mM after 90 minutes of growth (37°C 0.5mM EDTA, 0mM IPTG and 37°C 0.5mM EDTA, 1mM IPTG). Basal expression of plasmid encoded Reg26 (37°C 0.5mM EDTA) or full-expression by adding 1mM IPTG (37°C 0.5mM EDTA, +IPTG) is sufficient to restore the WT phenotype in cells lacking a chromosomally encoded Reg26 (Δ*cutC* pReg26). The average of three experiments with standard deviations is shown. (B) The viability of each strain shown in panel A; condition 37°C 0.5mM EDTA, +IPTG, both prior to (Before 0.5mM EDTA) and after growth in a final concentration of 0.5mM EDTA (120 minutes after 0.5mM EDTA), as determined by calculating the cfu/ml by plating on LB. Loss of pReg26 (Δ*cutC*) resulted in reduced cfu/ml LB when compared to the WT (WT) or complemented strain (Δ*cutC* pReg26). This reduction in cfu/ml corresponds to the observed decrease in OD600 (A). The cfu/ml data shown is shown as the $\log_2$ fold change relative to its genotype prior to the addition of EDTA. The average of three experiments with standard deviations is shown.

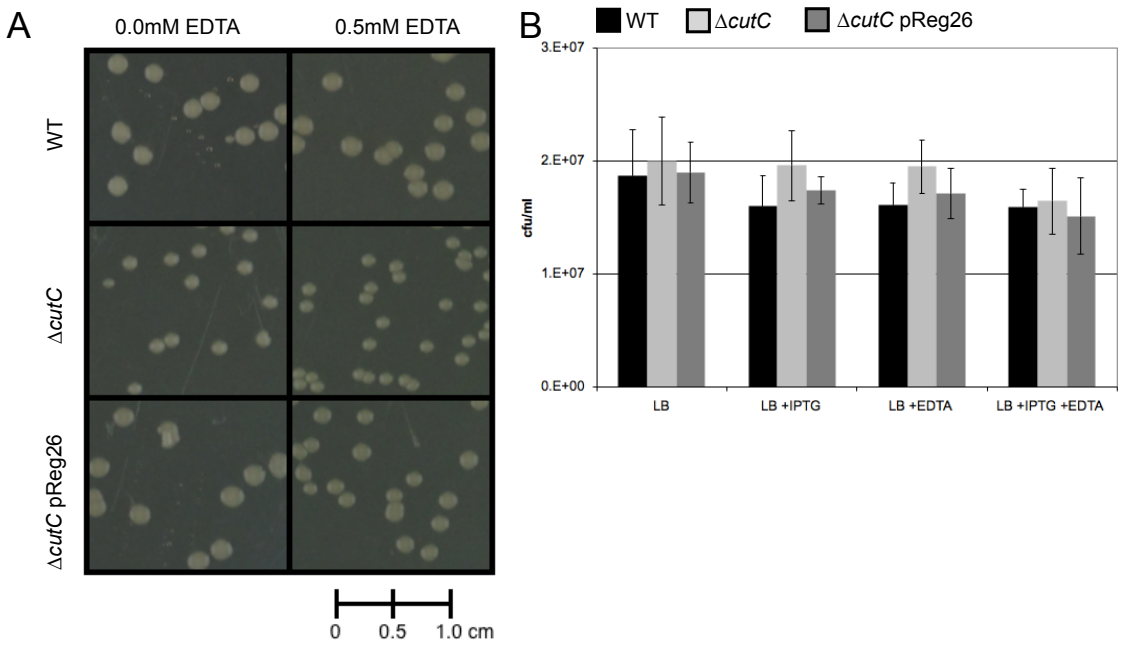

Figure 5. Loss of Reg26 results in a growth defect on solid media that is independent of EDTA exposure

Figure 5. Loss of Reg26 results in a growth defect on solid media that is independent of EDTA exposure

(A) A strain lacking Reg26 (Δ*cutC*) has a smaller colony size when compared to WT strains or those complemented for Reg26 (Δ*cutC* pReg26). This phenotype is independent of whether or not the cells are grown on solid media with EDTA. Cells were grown to an OD600 = 0.1, then plated on the solid media indicated and incubated for 16 hours at 37°C before photographing to determine colony size.

(B) The presence or absence of Reg26 does not affect cell viability during culture on solid media, regardless of whether or not EDTA is present, as indicated by the roughly equal cfu/ml of all strains in the conditions tested. Cells were grown as in (A) and plated as indicated (+IPTG; plates contain 1mM for induction of Reg26, +EDTA; plates contain 0.5mM EDTA) to determine cfu/ml. The average of three experiments with standard deviations is shown.

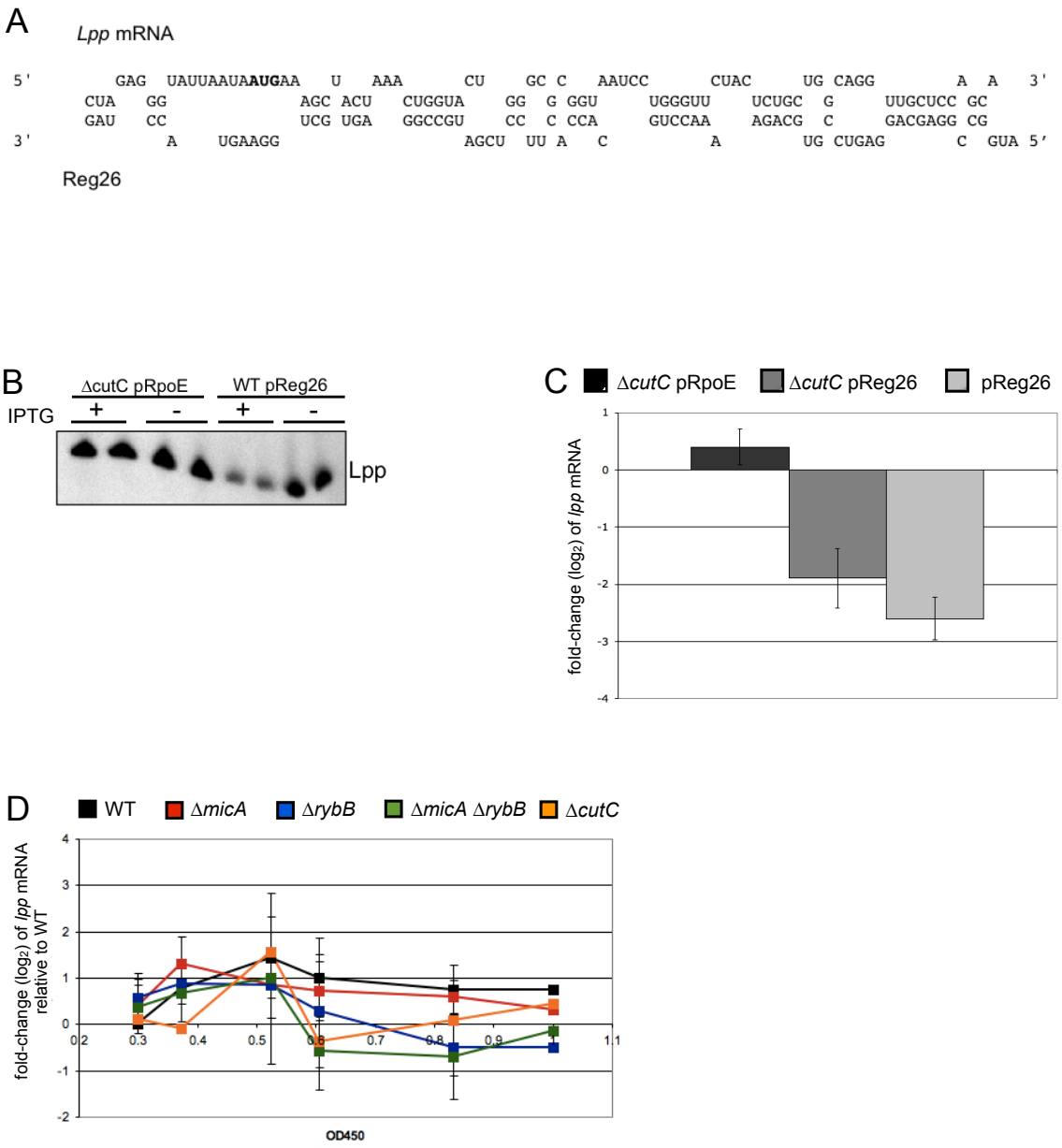

Figure 6. Reg26 targets the mRNA of the lipopotein Lpp for downregulation

Figure 6. Reg26 targets the mRNA of the lipopotein Lpp for downregulation

Expression of the sRNA Reg26 specifically downregulates the abundance of Lpp mRNA. (A) Base-pair interactions between the *lpp* mRNA and Reg26 as predicted by the *RNAhybrid* algorithm (42). The translational start site in Lpp is in bold.

(B) Northern blot showing that overexpression of σ^E in the absence of Reg26 results in no change in the transcript abundance of Lpp with (Δ*cutC* pRpoE, +) or without (Δ*cutC* pRpoE, -) induction of RpoE. However, if Reg26 is specifically expressed (WT pReg26, +) levels of *lpp* transcript is significantly reduced when compared to samples without induction of Reg26 (WT pReg26, -). Strains were grown overnight at 30°C in minimal media with glucose, diluted to OD450 = 0.03 in fresh minimal media with either glucose and grown to OD450 = 0.3. At this point, a pre-induction ('-',time=0) sample was harvested, cultures were induced with 1mM IPTG, and a 20 min. post-induction sample was harvested ('+', time=20'). RNA was prepared and transcript abundance of *lpp* assayed by Northern Blot (B) and cDNA was prepared and assayed by qRT-PCR (C). (C) Transcript abundance of *lpp* mRNA was quantified relative to time=0 of its own genotype and are plotted as $\log_2$ fold change (time 20/time 0). (D) Transcript abundance of *lpp* mRNA was quantified relative to WT at OD450=0.3. B-C The average of three experiments with standard deviations is shown.

Table 1.

A. Most Advanced Candidate sRNAs

Name	Predicted Promoter[a]	Hfq dependent[b]	5' start[c]	3' stop[c]	Terminator[d]	Length, nt	Northern Confirmed
reg26 (Co)	YES	YES	1956771	1956555	1956472	299 (T), 216 (L)	YES
reg120	NO	N/A	N/A	4169936	4169962	380	YES
reg63	NO	YES	N/A	1806103	1805841	280, 180, 100	YES
reg80	NO	YES	2723856	2724082	NP	226	YES

B. Advanced Candidate sRNAs

Name	Predicted Promoter[a]	Hfq dependent[b]	5' start[c]	3' stop[c]	Terminator[d]	Length, nt
reg69	NO	YES	2441626	2441946	NP	320
regminus15 (Co)	NO	NO	1321296	1321182	NP	114

C. Candidate sRNAs

Name	Predicted Promoter[a]	Hfq dependent[b]	5' start[c]	3' stop[c]	Terminator[d]
reg0	YES	NO	N/A	53260	NP
reg7	YES	NO	197590, 197802	N/A	NP
reg117	NO	N/A	N/A	4145387	NP
reg107	YES	NO	N/A	3784728	NP
reg46	NO	NO	986441	N/A	986631

[a] σ^E dependent promoter predictions courtesy of Virgil Rhodius, unpublished data
[b] Hfq dependence courtesy of Aixia Zhang from the Storz Lab, unpublished data
[c] As mapped by 5' and 3' RACE, the farthest 3' end mapped is given. In the case where multiple 5' starts exist all are listed
[d] Position of terminator if predicted by TransTermHP

(NP) Not Predicted
(Co) sRNA is on the complement strand
(T) is length from 5' start to the terminator predicted by TransTermHP
(L) is the length from 5' start to longest mapped 3' stop
^ This region gives a significant tiling feature when either sigma32 or sigmaE are overexpressed.

Table 2.

Component	Test Condition at 30˚C and 37˚C in LB broth and LB agar		
NaCl	300mM		
EDTA	0.1mM	1.0mM	0.5mM
Fusidic Acid	20ug/ml	40ug/ml	200ug/ml
SDS	0.50%	0.10%	1%
1% SDS with EDTA	0.1mM EDTA	0.5 mM EDTA	1.0 mM EDTA
Mecillinam	0.09ug/ml	0.36ug/ml	
Bile Salts	0.10%		
Nickel	1.0mM		

CHAPTER 3

Regulation of the Cell Envelope and the σ^E Stress Response by MicA and RybB

I. Background

A handful of sRNAs have already been discovered which regulate outer membrane proteins, and analysis of the half-life of eight mRNAs, many that encode for important constituents of the outer membrane, concluded that increased transcript degradation is occurring in a σ^E and Hfq dependent manner (16). In addition, hfq- strains have increased σ^E activity as demonstrated by the significant induction of σ^E regulon members. As σ^E activity reflects the level of unassembled porin monomers (49, 50, 69) it is likely that sRNAs are major regulators of porin production during steady state growth. Indeed, only two σ^E regulated sRNAs have been discovered so far, MicA and RybB. Our current understanding is that MicA and RybB act during slow growth (ppGpp activation of σ^E; see below) and during membrane stress (OMP activation of σ^E) to manage the abundance of OMPs, but studies have only shown their importance during transition to stationary phase (12, 15, 17, 39). Previously, low levels of σ^E activity are known to cause defects in the cell envelope and a loss of cell viability (37), suggesting that the cell must balance the activity level of σ^E in order to properly maintain the cell envelope.

One form of σ^E activation comes from accumulation of the alarmone ppGpp (70). This growth dependent regulation of σ^E is thought necessary to ready the cell for entry into non-growth conditions (71). In addition, σ^E is activated by the accumulation of misfolded proteins in the periplasm, and the best understood activators of this kind are outer membrane porins (72). The misfolded outer membrane porins activate a proteolytic

cascade, which includes RseP, that ultimately allows σ^E to complex with RNAP. The necessity of the proteolytic cascade to activate σ^E hinges on the abundance OMPs, most significantly OmpA and OmpC. One study found that among the OMP deletions tested, only the ompA-ompC double deletion removed the essentiality of the protease RseP (73). This indicates that the status of OMPs in the cell envelope is linked through σ^E activity to the viability of the cell.

There are several features of sRNAs that make them ideal regulators of OMPs. Indeed, their unique properties have already been highlighted in studies of quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*, which use four functionally redundant sRNAs to create an ultrasensitive regulatory switch (74). sRNAs' mRNA degradation capacity can provide the post-transcriptional regulation which is necessary to rapidly alter porin production (16) and their capacity for hierarchical cross-talk (71) enables integrated control of porin content. This hierarchy provides a way to regulate porins in a condition specific manner, and the rapid destruction of the sRNA during the regulatory process can provide fine temporal regulation of OMP abundance (75, 76). Finally, sRNAs can act on multiple targets to coordinate the regulation of many processes.

II. Results and Discussion

RybB and MicA are important for normal σ^E activity

We sought to determine in a natural context, without σ^E overexpression, the contribution of each sRNA to σ^E activity. As OmpC overexpression was previously found to activate σ^E (57), it suggested that target regulation by at least RybB, if not MicA, would be

important for maintaining normal σ^E activity. As indicated by previous work (77) WT strains have increasing levels of σ^E (Figure 1, Figure 2).

In strains missing either RybB, MicA, or both, σ^E activity can vary significantly from that of WT (Figure 1A, Figure 2). Previously it has been shown in stationary phase *Salmonella* cultures that MicA and RybB are important for normal σ^E activity, and in the absence of both sRNAs σ^E activity is increased synergistically (12). Our data show that this is not the case in *E.coli,* as the absence of both MicA and RybB (Δ*micA* Δ*rybB)* causes an additive effect on σ^E activity level in stationary phase (Figure 2B). Moreover, MicA and RybB contribute equally to σ^E activity during exponential growth through early stationary phase (OD450 = 0.3-0.6), as the Δ*micA* and Δ*rybB* strains have similar levels of *rpoE* mRNA (Figure 1), and these levels are typically higher than that of the WT strain. Interestingly, the contribution of MicA σ^E to declines into stationary phase, as the WT and Δ*micA* strains exhibit almost identical levels of rpoE mRNA as the OD450 increases.

RybB and MicA have a significant impact on the abundance of key OMP transcripts

To characterize the importance of the interaction of MicA and RybB with their previously described OMP targets (12, 14, 17, 66) we assessed OMP transcript abundance throughout growth in WT, Δ*micA*, Δ*rybB* and Δ*micA* Δ*rybB* strains by qRT-PCR (Figure 2). To determine the contribution of each sRNA to target regulation we compared the relative transcript abundance of each target in strains with both sRNAs present (WT) or missing one (Δ*micA,* Δ*rybB*) or both of the sRNAs (Δ*micA* Δ*rybB*). As σ^E is most active in stationary phase we compared the relative transcript abundance of

each target at early stationary phase (time 180, OD450=0.9). Our results generally confirm previous overexpression studies (12, 17, 66).

As expected in WT cells, all targets (*ompF, tsx, fiu, ompX, ompA* and *ompC*) are downregulated as σ^E activity increases as measured by the abundance of *rpoE* transcript (Figure 2A). However, only *ompF*, *tsx* and *fiu* transcript abundance is inversely proportional to σ^E level overtime. For *ompX*, *ompA* and *ompC*, transcript levels initially increase, followed by a slight decrease.

Those transcripts that are negatively regulated (green trends; *ompF*, *tsx*, *fiu*) are not greatly affected when either sRNA is absent. This suggests that the sRNAs play a smaller role under the conditions tested, and that the majority of transcript downregulation is due to other factors. This is not surprising as the σ^E response may occur in tandem with other regulatory features such as those controlled by EnvZ-OmpR, PhoP-PhoQ, or CpxA-CpxR.

As expected, dysfunctional regulation of transcript abundance is observed for *tsx*, *fiu*, and *ompC*. MicA and RybB both downregulate *tsx*, and subsequently only the strain missing both sRNAs (Δ*micA* Δ*rybB*) has reduced capacity to downregulate *tsx* (Figure 2A, 2C). Likewise, the transcript of *fiu* is downregulated by RybB, and either strain missing RybB (Δ*rybB* and Δ*micA* Δ*rybB*) show a reduced capacity to downregulate *fiu*. Without RybB present to downregulate *ompC* (Δ*rybB* and Δ*micA* Δ*rybB*), levels of *ompC* transcript abundance are severely upregulated throughout growth in comparison to the WT strain. Moreover, the trend noted for *ompC* in Figure 2A, B throughout growth is clearly visibly at early stationary phase, as levels of *ompC* in strains missing RybB (Δ*rybB*, Δ*micA* Δ*rybB*) are elevated 4-fold when compared to WT (Figure 2C).

Unexpected dysfunctional regulation of transcript abundance was observed for *ompF*, *ompA*, and *ompX*. Cells missing only MicA or RybB (ΔmicA or ΔrybB) but not both (ΔmicA ΔrybB) had a reduced capacity to downregulate *ompF* at earlier time points (times 0, 30, 90, and 120), by early stationary phase were not significantly different than WT (Figure 2B), but were significantly different later in stationary phase (time 240).

OmpA is a joint target of both MicA and RybB, and as expected, has a slightly reduced capacity to be regulated when both MicA and RybB are absence (ΔmicA ΔrybB). However, when RybB alone is absent (ΔrybB), greater than expected downregulation of *ompA* transcript abundance (times 90, 120, 180 and 240). Similarily, *ompX* which is targeted by MicA, also shows greater than expected downregulation of transcript abundance (times 120, 180 and 240) when RybB alone is absent (ΔrybB). However, strains lacking MicA (ΔmicA) have a reduced capacity regulate *ompX*, whereas they do not have a reduced capacity to regulate *ompA*.

Our data indicate that MicA provides sufficient σ^E dependent regulation of *ompX*, and that CyaR makes almost no contribution to the σ^E dependent regulation of *ompX* in the conditions tested (Figure 3A; growth with glucose present, 3B; growth with maltose present). Moreover, CyaR plays no role in the regulation of targets tested that are regulated by both RybB and MicA (*ompA, ompF*, *tsx*) or just RybB (*nmpC*).

MicA and RybB make significant contributions σ^E activity that may ultimately govern cell survival during cell envelope stress

The σ^E response is engaged to promote cell survival and maintain homeostasis in the cell envelope. As RybB and MicA target OMPs that are known to affect σ^E activity

(66) we sought to determine the effects of increased expression of RybB and MicA on σ^E activity.

We found that both the kinetics and amplitude of σ^E activity were markedly different in strains with increased expression of RybB or MicA (Figure 4). Wildtype cells display an increase in σ^E activity overtime (WT; differential rate of 3.16) whereas expression of either sRNA decreases or reduces σ^E activity to very low levels (respectively pMicA; differential rate 1.73 and pRybB; differential rate 0). When compared to wildtype cells the amplitude of σ^E activity is at least 2-fold and 7-fold lower in cells overexpressing MicA (pMicA) and RybB (pRybB), respectively.

Previously, it was found that the growth-phase dependent increase in σ^E activity can be abolished by expression of the anti-sigma factor RseA, and RseB, a protein that binds to RseA, promoting the negative regulation of σ^E (37). Strikingly, that same low level of σ^E activity is achieved through overexpression of RybB (pRybB). Moreover, RybB is clearly more effective at lowering σ^E activity than MicA. This may be due to RybB's action on targets that are known to activate σ^E, such as *ompC*, or other targets that are uniquely regulated by RybB and not MicA (66). Moreover, this data indicate that the inducing signal makes significant contributions to σ^E activity, in addition to contributions from sigma factor competition. This may have significant consequences for how homeostatic control of σ^E activity is achieved by the cell.

This evidence that either MicA or RybB can reduce the level of σ^E activity to levels known to be low enough to kill the cell, and yet the cell survives, suggested that MicA and RybB may provide a mechanism for the cell to escape death. Previous work had shown that overexpression of either MicA or RybB is sufficient to rescue the cell

from death during low levels of σ^E (19). To gain insight into how this is occurring, we tested whether MicA or RybB could rescue the cell from death if the cell was also challenged with additional envelope stress. Our results indicate that under a variety of different temperatures (25°C, 30°C, 37°C) or when grown at 30°C in the presence of 0.1mM EDTA, strains overexpressing either RybB or MicA behave identically whether or not σ^E activity has been shutoff (pRseAB containing strains have shutoff σ^E activity). However, at 42°C, or when grown at 30°C in the presence of 0.1% SDS or 300mM NaCl, overexpression of either sRNA is not enough, and the viability of all strains where σ^E activity has been shutoff is very similar.

These findings led us to examine the necessity of RybB for growth during SDS mediated stress. We tested strains that are capable of activating σ^E for their ability to grow in liquid media in the presence of 1% SDS. These strains were either fully wildtype (WT), missing RybB (Δ*rybB*), or only had RybB under the control of an IPTG inducible promoter, pTrc99a (Δ*rybB* pRybB). During regular growth in LB, the strain missing RybB has a slight growth defect that becomes exacerbated as the cells are challenged with 1% SDS. IPTG induced expression of RybB slightly restores growth, regardless of whether the expression is at a low level (0mM IPTG; pTrc99a is known to be slightly active in with no IPTG present), or maximally induced (1mM IPTG). This suggests that while expression of RybB is necessary for the cell to grow normally in the presence of 1% SDS, the exact level and timing of RybB expression is important to fully rescue the cells.

III. Figures

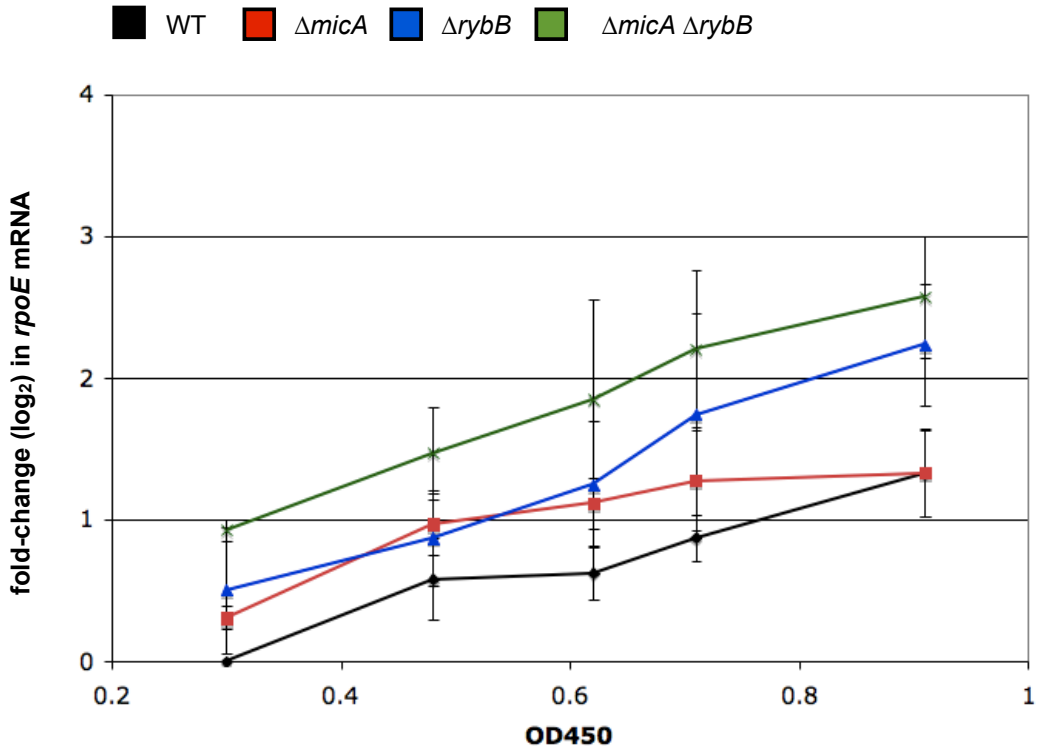

Figure 1. Loss of MicA or RybB contributes significantly to σ^E activity

Figure 1. Loss of MicA or RybB contributes significantly to σ^E activity

In the absence of RybB (Δ*rybB* and Δ*micA* Δ*rybB*) the abundance of *rpoE* mRNA is increased relative to a WT strain; this effect is not as significant in the absence of just MicA (Δ*micA*). (B) In a WT strain *rpoE* transcript abundance is a good measure of σ^E activity and has a similar transcriptional profile with transcripts of other σ^E dependent genes. Bacteria grown overnight in minimal media with glucose and subcultured to OD450 = 0.03 into fresh media and grown at 30°C. cDNA was prepared and transcript abundance assayed by qRT-PCR as described in *Materials and Methods.* The average of three experiments with standard deviations is shown. Data is relative to WT at OD450 = 0.3.

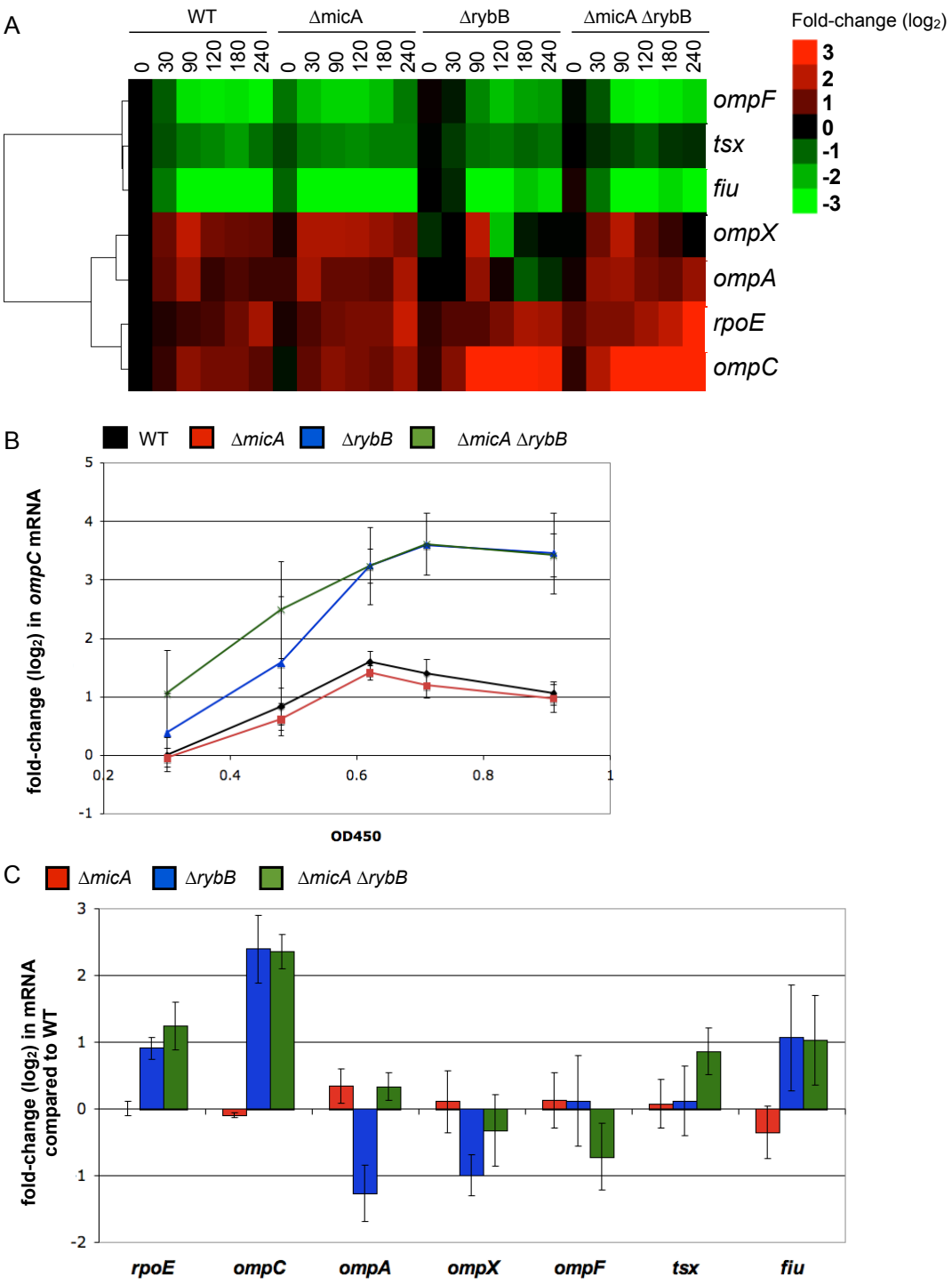

Figure 2. Loss of target regulation by MicA and RybB contributes significantly to the σ^E response

Figure 2. Loss of target regulation by MicA and RybB contributes significantly to the σ^E response

Wildtype (WT) strains or strains missing either MicA (ΔmicA) RybB (ΔrybB) or both (ΔmicA ΔrybB) were assessed for the abundance of the indicated OMP transcripts from early exponential phase growth (time 0 minutes, '0') throughout growth until early stationary phase (time 240 minutes '240').

(A) Times indicated are from OD450 = 0.3 (0 minutes) to OD450 = 1.1 (240 minutes later). A representative experiment is shown as a heat map.

(B) Comparison of the transcript abundance to the WT strain in early exponential phase growth (OD450=0.3) for *ompC* mRNA. The average of three experiments with standard deviations is shown.

(C) Comparison of transcript abundance to the WT strain during early stationary phase (at time 180 minutes). The average of three experiments with standard deviations is shown. Bacteria grown and assayed as described for Figure 1.

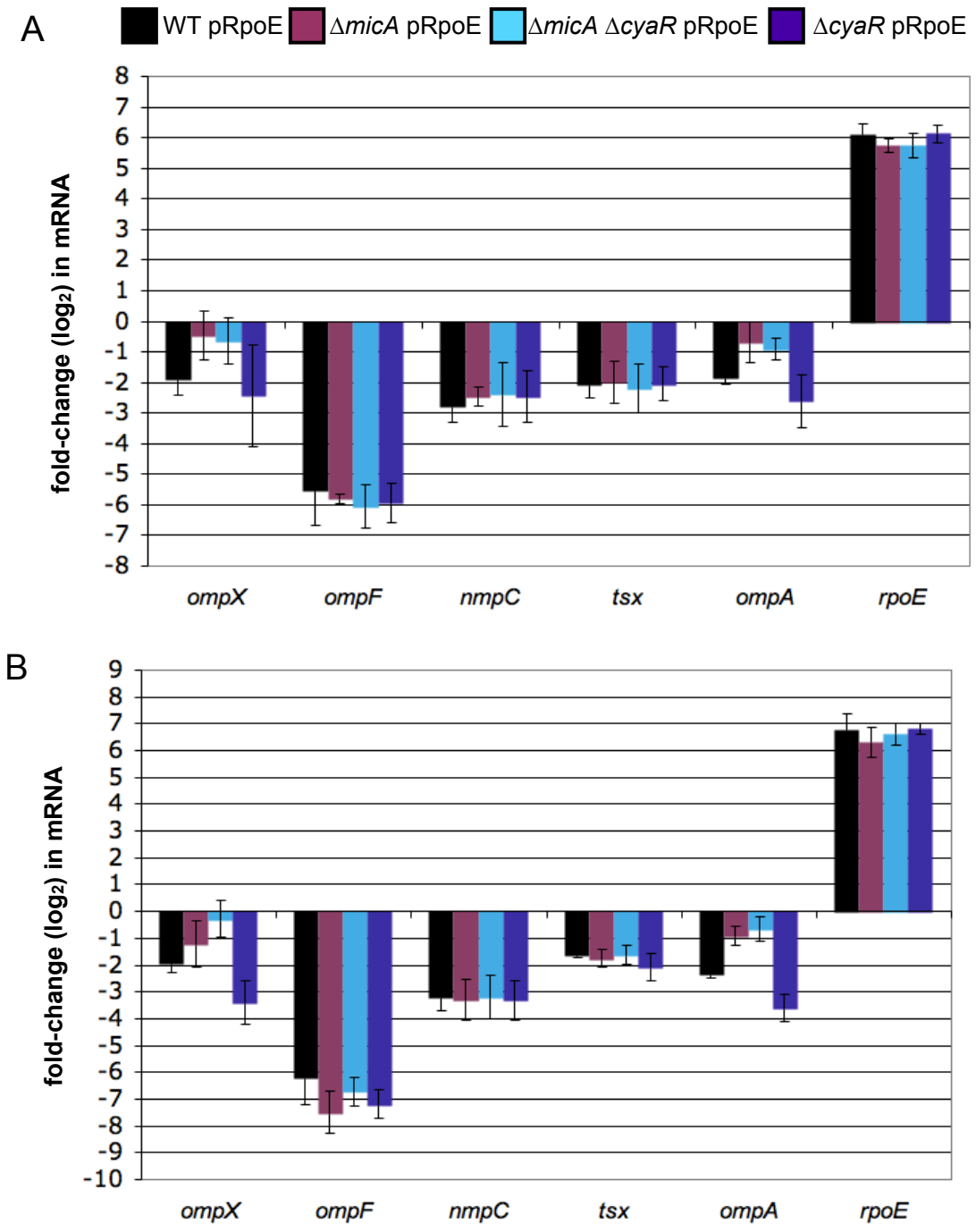

Figure 3 Additional σ^E dependent regulation of OmpX is not mediated by CyaR.

Figure 3 Additional σ^E dependent regulation of OmpX is not mediated by CyaR.

Wildtype strains with overexpression of *rpoE* from a plasmid (WT pRpoE) or strains missing either MicA (Δ*micA* pRpoE) CyaR (Δ*cyaR*) or both (Δ*micA* Δ*cyaR*) were assessed for the abundance of the indicated OMP transcripts. Bacteria grown overnight at 30˚C in either glucose minimal media or maltose minimal media and were diluted to OD_{450} = 0.03 in fresh minimal media and grown at 30°C to OD450 = 0.3. At this point, a pre-induction (time=0) sample was harvested, cultures were induced with 1mM IPTG, and a 20 min. post-induction sample was harvested (time=20'). The fold-change is the $\log_2$ ratio of these samples.

(A) Grown in minimal media with glucose present

(B) Grown in minimal media with maltose present

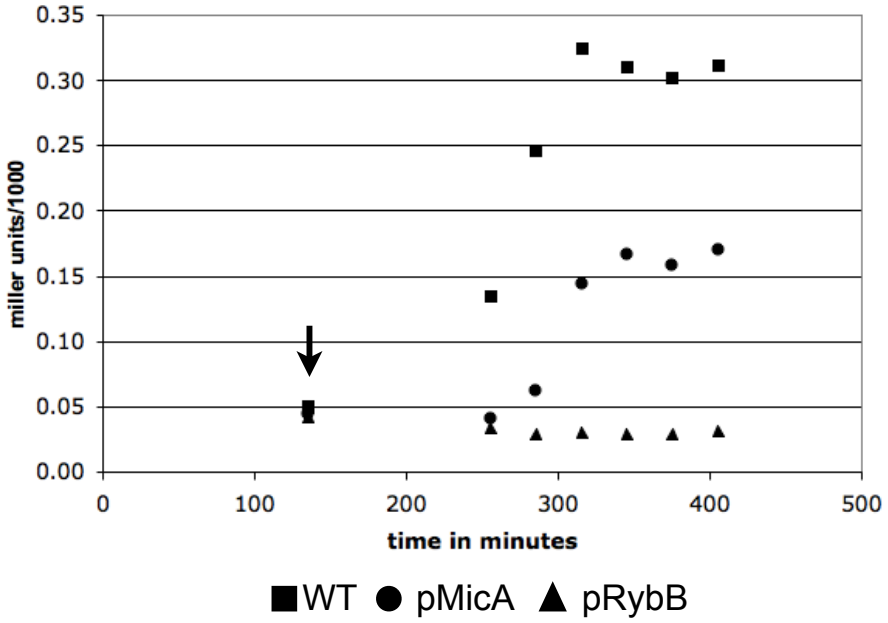

Figure 4 Overexpression of either RybB or MicA affects σ^E activity throughout growth

Figure 4 Overexpression of either RybB or MicA affects σ^E activity throughout growth

σ^E activity of each strain (WT or sRNA ovexpression strains; pMicA or pRybB) measured from exponential to stationary phase cultures. σ^E activity 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 OD600 = 0.03 in fresh media and grown at 30°C. 1mM IPTG was added just before 135 minutes of growth (OD600 ~0.1) to induce overexpression of a sRNA if indicated (pMicA or pRybB) as indicated by the arrow. A representative experiment is shown.

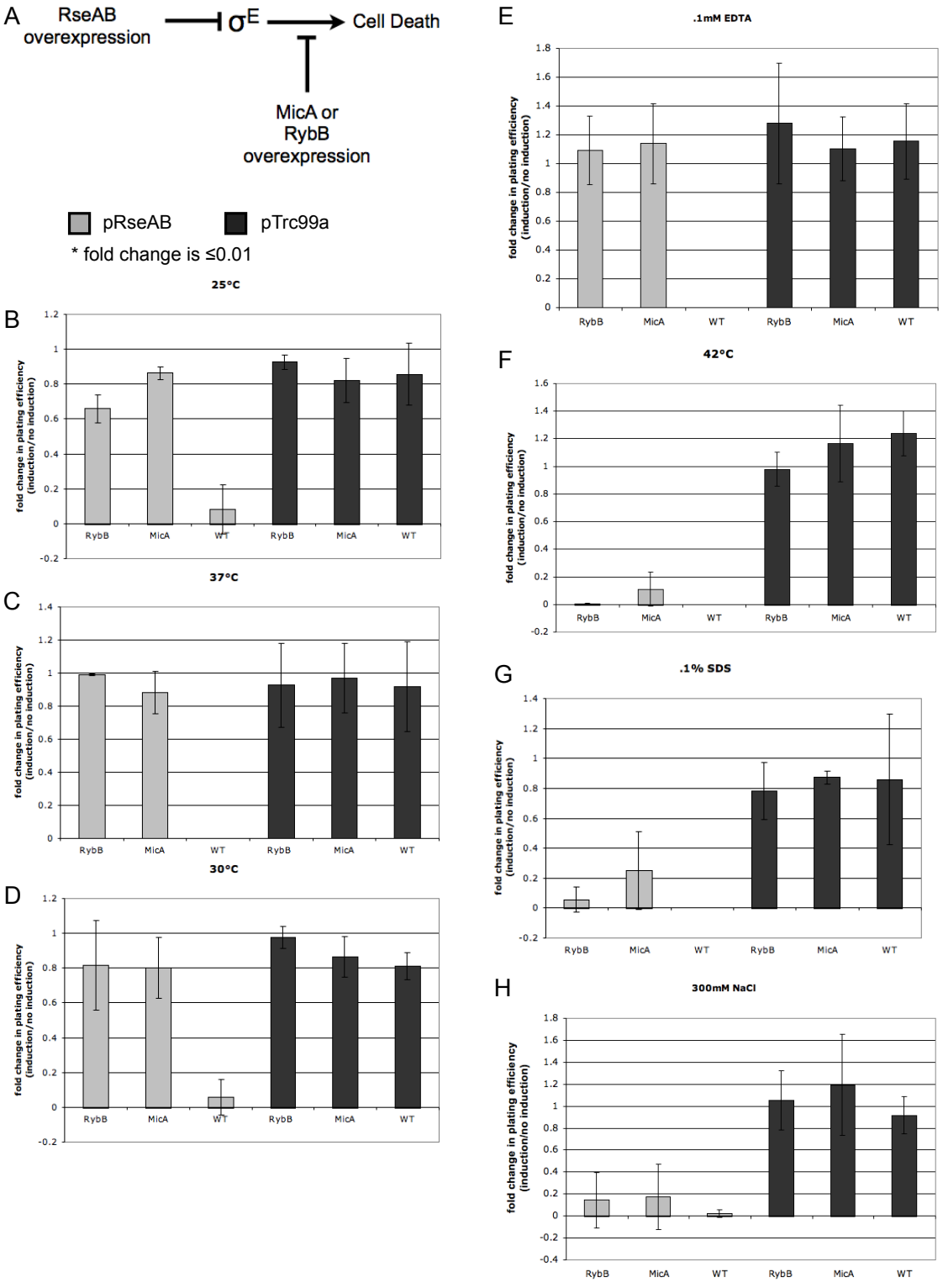

Figure 5 Expression of either MicA or RybB rescues cells with low activity σ^E from death during some cell envelope stresses

Figure 5 Expression of either MicA or RybB rescues cells with low activity σ^E from death during some cell envelope stresses

When σ^E is held inactive by the overexpression of its negative regulators, RseA and RseB, growth ceases prematurely and viability decreases (A). Under some cell envelope stress inducing conditions the overexpression of MicA or RybB is sufficient to rescue the cell from death (B). Cell viability was measured by fold change in plating efficiency (cfu/ml) of strains harboring various plasmids with IPTG inducible promoters; overexpression of RseA/B from the σ^E shutoff plasmid (pRseAB) or empty vector (pTrc99a) without (WT) or with concomitant overexpression of the plasmid-encoded sRNA (RybB or MicA) were compared. *rseAB* as well as MicA and RybB are controlled by IPTG inducible promoters. Bacteria were grown as described in Figure 1, until an OD450 = 0.3 was achieved. Bacteria were then serially diluted and plated with 1mM IPTG (induction plate) or without IPTG (no induction plate) to determine the relative fold change in plating efficiency per condition. Conditions are as follows: all plates were kept at 30°C unless indicated otherwise (25°C, 37°C, 42°C) and contained the appropriate antibiotics. Per condition when indicated the plates also contained the following: .1% SDS, 300mM NaCl, and .1mM EDTA. The average of three experiments with SD is shown.

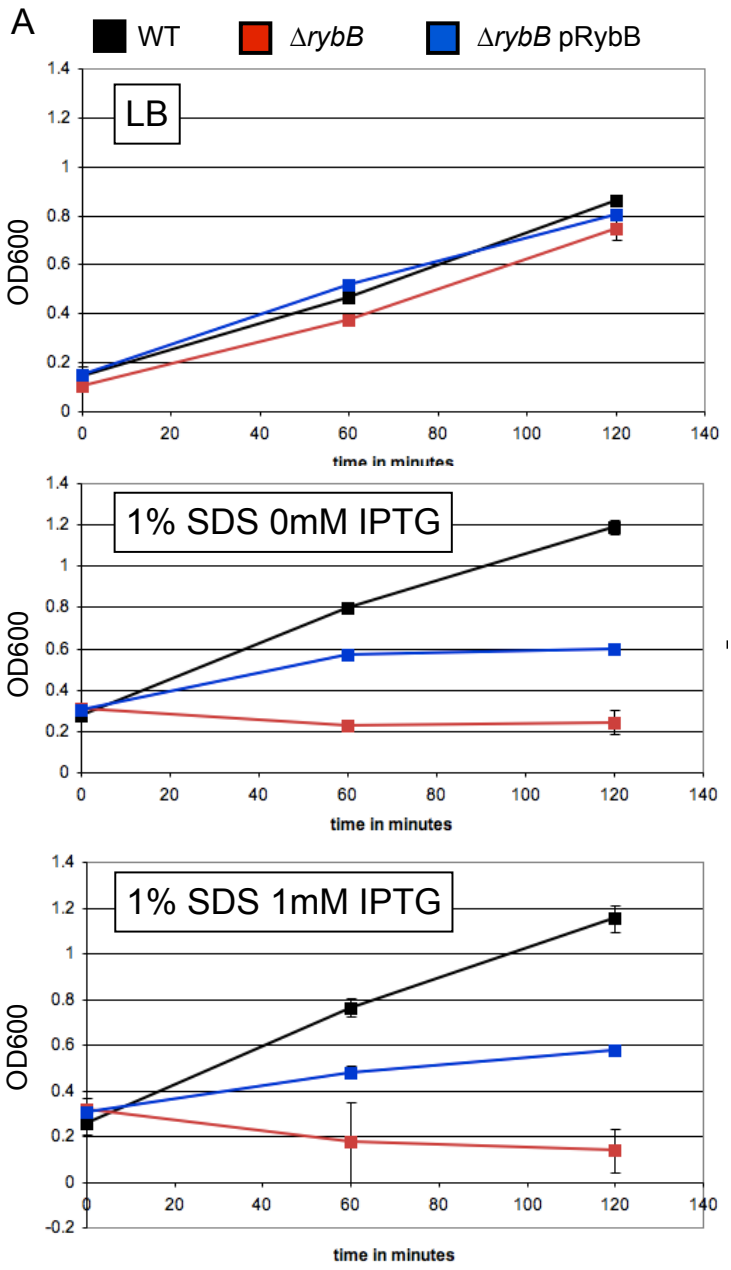

Figure 6 RybB is important for growth during SDS mediated stress

Figure 6 RybB is important for growth during SDS mediated stress

Wildtype (WT) strains or strains missing RybB (Δ*rybB*) or missing RybB with RybB expressed from a plasmid (Δ*rybB* pRybB) were assessed for their ability to grow in a variety of membrane stress inducing conditions. Growth overtime was measured by OD600. pRybB contains a IPTG inducible promoter (pTrc99a) driving the expression of RybB. 1mM IPTG induces maximal expression of RybB from the plasmid. The average of three experiments with standard deviations is shown.

Table 1 Strains and plasmids for Chapter 2 and 3 that were not included in Table S3

Common name	Database #	Comment	Reference
Reg26-*gfp* fusion	CAG62789	reg26plus5::gfp; Reg26 promoter cloned through +5 of Reg26 fused to GFP using BamHI and XhoI sites in PUA66. For GFP promoter strength assays.	this work
ΔcutC	CAG62124	ΔcutC from the Keio collection CAG strain collection #59	this work
ΔcutC pRpoe	CAG62203	CAG62124 and CAG25197	this work
ΔcutC ptrc99a	CAG62205	CAG62124 and CAG25196	this work
ΔcutC pReg26	CAG62209	CAG62124 and CAG62154	this work
ΔcyaR	CAG62212	ΔcyaR	this work
ΔmicA ΔcutC pRpoE	CAG62214	ΔcyaR in CAG62130	this work
ΔmicA ΔcutC ptrc99a	CAG62216	ΔcyaR in CAG62131	this work
ΔcyaR ptrc99a	CAG62218	ΔcyaR in CAG25196	this work
ΔcyaR pRpoE	CAG62220	ΔcyaR in CAG25197	this work
ΔmicA pRpoE	CAG62130	CAG62031 and CAG25197	this work
ΔmicA ptrc99a	CAG62131	CAG62031 and CAG25196	this work
pReg26	CAG62154	ptrcEGoperator-reg26	this work
ΔrybB pRybB	CAG62194	CAG62150 in CAG62002	this work

Table 2 Primers, probes and oligonucleotides for Chapter 2 and 3 that were not included in Table S4

Primer Name	Sequence	Use	Comment
Lpp_1_14	\5Biosg\TAGCAGCCTGAACGTCGGAACGCATTGCGT	Probe	For Probing Northern Blots
N_reg120	\5Biosg\CCGGCGGATTTGTCCTACTCAGGAGAGCGTT	Probe	For Probing Northern Blots
N_reg63	\5Biosg\TCGATTTCGCCTTCACCTAACTGCTCGCTCA	Probe	For Probing Northern Blots
N_reg80	\5Biosg\ACGGATACGGCTTCCCCAACTTGCCCACTTC	Probe	For Probing Northern Blots; labeled tube is 'N_plus44'
N_reg26	\5Biosg\ATGGACTTCCAGCACTCCGGCATCGAGGAAG	Probe	For Probing Northern Blots
3'gsp2_plus20	AGTACGGCTTCGATCAGGAA	Primer	For 5' or 3' RACE
3'gsp2_plus43	GTGTATGCGGCGTTAAAGCA	Primer	For 5' or 3' RACE
3'gsp2_plus67	TTTGTGTTCGTGGTCGTGTT	Primer	For 5' or 3' RACE
3'gsp2_plus69	TGGTGATGGACGTGAAAGTG	Primer	For 5' or 3' RACE
3'gsp2_plus45	GGTTGCCAGGGTGAAGTTGG	Primer	For 5' or 3' RACE
3'gsp2_minus53	CTCCAGTACTTTGATGGACGC	Primer	For 5' or 3' RACE
3'reg0	GCATGCTGATGAACCGTAAG	Primer	For 5' or 3' RACE
3'reg7	GTTATCGCATTGGCTCGATT	Primer	For 5' or 3' RACE
3'reg15	ACCTGTGAAAGGCGCTAAAA	Primer	For 5' or 3' RACE
3'reg117	GGCATACCTGGTGGCAGTAA	Primer	For 5' or 3' RACE
3'reg120	CGCTCTCCTGAGTAGGACAAA	Primer	For 5' or 3' RACE
3'reg130	TTAAGTGCGATGGGACTGCT	Primer	For 5' or 3' RACE
3'reg61	GCGTGAACTGGCGATATCAT	Primer	For 5' or 3' RACE
3'reg68	GGTACGCACGGCGAAAAC	Primer	For 5' or 3' RACE
3'reg69	GGGTAATCTGCTCTCGCAGT	Primer	For 5' or 3' RACE
3'reg80	GAGGAGCTTAAGGATAAATTTCTGG	Primer	For 5' or 3' RACE
3'regminus15sE	GCTGTGCAGGTCGTAAATCA	Primer	For 5' or 3' RACE
3'reg107	GGATCACTGCGTGGAAGAAG	Primer	For 5' or 3' RACE
3'reg63	AGCAGTTAGGTGAAGGCGAA	Primer	For 5' or 3' RACE
3'reg126	GACCAGCGTCAGCACCGTAA	Primer	For 5' or 3' RACE
3'reg26	TCACCGATGCGTTATCGTAA	Primer	For 5' or 3' RACE
gsp1_26	GATGGCGTTCAATGATTCCT	Primer	For 5' or 3' RACE
gsp1_1	AGTGTTCCAGCAATGCTTGC	Primer	For 5' or 3' RACE
gsp1_15	GTCGCTATTTTTAGCGCCTTTC	Primer	For 5' or 3' RACE
gsp1_46	TGTAAATATCATCACGTCTCTATG	Primer	For 5' or 3' RACE
gsp1_117	CACCAGGTATGCCATTTTAACC	Primer	For 5' or 3' RACE
gsp1_118	TGTTCACGGCGATACCAGTA	Primer	For 5' or 3' RACE
gsp1_130	ATAACCACCACCGCCAACAT	Primer	For 5' or 3' RACE
gsp1_120	AACGTTCAAATCCGCTCCCG	Primer	For 5' or 3' RACE
gsp2_26	TTACGATAACGCATCGGTGA	Primer	For 5' or 3' RACE
gsp2_1	GCTTGCATAATGTGCCTGTC	Primer	For 5' or 3' RACE
gsp2_15	CGCTATTTTTAGCGCCTTTCACA	Primer	For 5' or 3' RACE
gsp2_46	TCATCACGTCTCTATGGAAATATG	Primer	For 5' or 3' RACE
gsp2_117	CACCAGGTATGCCATTTTAACCT	Primer	For 5' or 3' RACE
gsp2_118	TCATTCGCAGTTGCAGTACC	Primer	For 5' or 3' RACE
gsp2_130	CCAACATCAGCAGTCCCATC	Primer	For 5' or 3' RACE
gsp2_120	TCCTACTCAGGAGAGCGTTCA	Primer	For 5' or 3' RACE
gsp1_0	GTTCATCAGCATGCGGTATG	Primer	For 5' or 3' RACE
gsp1_35	CCACTTCGCCTTTGTACAGA	Primer	For 5' or 3' RACE
gsp1_37	GTAATGGCATCAGACGCTGA	Primer	For 5' or 3' RACE
gsp1_42	TAACCGCTTCTACCTGGTCG	Primer	For 5' or 3' RACE
gsp1_7	AATCGAGCCAATGCGATAAC	Primer	For 5' or 3' RACE
gsp1_63	TTCGATTTCGCCTTCACCTA	Primer	For 5' or 3' RACE
gsp2_0	GCGGTATGCACGATCTTTCT	Primer	For 5' or 3' RACE
gsp2_35	CTTCGCCTTTGTACAGACCA	Primer	For 5' or 3' RACE
gsp2_37	CGCTCAGCAGAACAAAATCA	Primer	For 5' or 3' RACE
gsp2_42	CTGGTCGTTTTCGTTCTCGT	Primer	For 5' or 3' RACE
gsp2_7	ATCGAGCCAATGCGATAACA	Primer	For 5' or 3' RACE
gsp2_63	TTCGCCTTCACCTAACTGCT	Primer	For 5' or 3' RACE
gsp1_90	CAGCTCTACCGTTTCGGCAA	Primer	For 5' or 3' RACE
gsp1_107	GCTTTCGCGACTTCTTCCAC	Primer	For 5' or 3' RACE
gsp1_108	TCCTTCTTTCCCTCCGGTAT	Primer	For 5' or 3' RACE
gsp1_109	TCACCGAAACTGATCGCTAA	Primer	For 5' or 3' RACE
gsp1_126	CTTACGGTGCTGACGCTGGT	Primer	For 5' or 3' RACE
gsp1_127	ACTTGCGGCAGCCAGTAACT	Primer	For 5' or 3' RACE
gsp2_90	AGCTCTACCGTTTCGGCAAA	Primer	For 5' or 3' RACE
gsp2_107	CTTCTTCCACGCAGTGATCC	Primer	For 5' or 3' RACE
gsp2_108	GCGATCCCTTGCTGAAAATA	Primer	For 5' or 3' RACE
gsp2_109	GAGCTGTCTTTGCGGAACAC	Primer	For 5' or 3' RACE
gsp2_126	TTACGGTGCTGACGCTGGTC	Primer	For 5' or 3' RACE
gsp2_127	CGGCAGCCAGTAACTCTCTT	Primer	For 5' or 3' RACE
gsp1_micC	TGTTGGAAAATCAGTGGCAA	Primer	For 5' or 3' RACE
gsp1_ryeC	AGCACGTCATCATACTGGTGA	Primer	For 5' or 3' RACE
gsp1_sroC	CGAAGATTGTTACCCAGCGT	Primer	For 5' or 3' RACE
gsp1_CsrB	TTCTCCATCCTGGAGGTGTC	Primer	For 5' or 3' RACE
gsp1_mgta 5'	AAATCGGCTGACCATCAAAG	Primer	For 5' or 3' RACE

gsp2_micC	CAATGGCCCAACAGAAAATA	Primer	For 5' or 3' RACE
gsp2_ryeC	TCCAGGCTTACTAAGAACACCA	Primer	For 5' or 3' RACE
gsp2_sroC	GAACGACCATCAGGCGTATAG	Primer	For 5' or 3' RACE
gsp2_CsrB	TCATCCTCTTCGCTTCATCC	Primer	For 5' or 3' RACE
gsp2_mgta 5'	AGCGTGTTGAAGCAGTGATG	Primer	For 5' or 3' RACE
gsp1_61	GGATGAATGATATCGCCAGTT	Primer	For 5' or 3' RACE
gsp1_68	GAATTCGTTTTCGCCGTGCG	Primer	For 5' or 3' RACE
gsp1_69	CAAACACTGCGAGAGCAGAT	Primer	For 5' or 3' RACE
gsp1_80	AGATTCAGCAACGGATACGG	Primer	For 5' or 3' RACE
gsp1_minus15	TTGAGCGACACGAATTATGC	Primer	For 5' or 3' RACE
gsp2_61	AATGATATCGCCAGTTCACG	Primer	For 5' or 3' RACE
gsp2_68	TTTTCGCCGTGCGTACCACA	Primer	For 5' or 3' RACE
gsp2_69	ACTGCGAGAGCAGATTACCC	Primer	For 5' or 3' RACE
gsp2_80	AACTTGCCCACTTCCATACG	Primer	For 5' or 3' RACE
gsp2_minus15	TGATTTACGACCTGCACAGC	Primer	For 5' or 3' RACE
gsp1_minus1	TCAGGTCCAGGAATGGAAAG	Primer	For 5' or 3' RACE
gsp1_minus13	CGAATTTGCTGGCAATCATAG	Primer	For 5' or 3' RACE
gsp1_ldhAreg	CGGCACGGTAGAACTTGATT	Primer	For 5' or 3' RACE
gsp1_prom4469	AATTCCAGCTCAAAGCCAAA	Primer	For 5' or 3' RACE
gsp1_minus32	AACGGCATGCGATGTACAATA	Primer	For 5' or 3' RACE
gsp1_plus3	GGTTCCGTATTTCGGTTTGA	Primer	For 5' or 3' RACE
gsp1_plus11	TTCCTTCGGGTTCGAGTATG	Primer	For 5' or 3' RACE
gsp1_plus20	CAAACCAGTACGGCTTCGAT	Primer	For 5' or 3' RACE
gsp1_plus32	TCACCTGTTGATCGTTCTGC	Primer	For 5' or 3' RACE
gsp1_plus43	TCTGCTTTAACGCCGCATAC	Primer	For 5' or 3' RACE
gsp1_plus44	AACTTGCCCACTTCCATACG	Primer	For 5' or 3' RACE
gsp1_plus47	CATCTCCACGAATCACAACG	Primer	For 5' or 3' RACE
gsp1_plus49	CCCGGAATCAGGATTAGTCA	Primer	For 5' or 3' RACE
gsp1_prom2127	GGTACTTCCGGTGGTAACGA	Primer	For 5' or 3' RACE
gsp1_prom2128	CTTGTGCAGCATCGGAAATA	Primer	For 5' or 3' RACE
gsp1_plus58	ATCGTACCGCCCACTAACAG	Primer	For 5' or 3' RACE
gsp1_plus67	GATAAACCACAGCGGGGTAA	Primer	For 5' or 3' RACE
gsp1_plus69	GCTACCCACTTTCACGTCCA	Primer	For 5' or 3' RACE
gsp1_rprA	TTTCACTCAGGGGATTTCCA	Primer	For 5' or 3' RACE
gsp1_gcvB	GGTCTGAATCGCAGACCAAT	Primer	For 5' or 3' RACE
gsp1_tp2	CCATCTGGCCTTTATCGAAG	Primer	For 5' or 3' RACE
gsp1_gadY	GGACCGGGAAGAGGATAGTC	Primer	For 5' or 3' RACE
gsp1_fimA3prime	CAAGCGGCGTTAACAACTTC	Primer	For 5' or 3' RACE
gsp1_plus19	TCATCGAGCAGTAATAGTTTCG	Primer	For 5' or 3' RACE
gsp1_plus22	AGCCAGTCGTTCGTTTTACC	Primer	For 5' or 3' RACE
gsp1_plus45	GCCAACTTCACCCTGGCAAC	Primer	For 5' or 3' RACE
gsp1_minus53	TTGCCGCGTCCATCAAAGTA	Primer	For 5' or 3' RACE
gsp2_minus1	ATGGTACTGGATGGCAAAGC	Primer	For 5' or 3' RACE
gsp2_minus13	GCTGGCAATCATAGTCACCTC	Primer	For 5' or 3' RACE
gsp2_ldhAreg	ATGCTGCCTAACACCTGGAT	Primer	For 5' or 3' RACE
gsp2_prom4469	CAAAGCCAAAGGACTCGTTC	Primer	For 5' or 3' RACE
gsp2_minus32	CGGCATGCGATGTACAATAC	Primer	For 5' or 3' RACE
gsp2_plus3	AATCTGCGCTTTCAGTTCGT	Primer	For 5' or 3' RACE
gsp2_plus11	CATGCACCCACTAAATGCAG	Primer	For 5' or 3' RACE
gsp2_plus20	AGTACGGCTTCGATCAGGAA	Primer	For 5' or 3' RACE
gsp2_plus32	CACGAAGATCGCTAACACCA	Primer	For 5' or 3' RACE
gsp2_plus43	TGCTTTAACGCCGCATACAC	Primer	For 5' or 3' RACE
gsp2_plus44	CCCACTTCCATACGTGTCCT	Primer	For 5' or 3' RACE
gsp2_plus47	TGGGCGTAAAACATCAGACA	Primer	For 5' or 3' RACE
gsp2_plus49	CGCTTCGCTGTCATCAATAA	Primer	For 5' or 3' RACE
gsp2_prom2127	CTTCCGGTGGTAACGACATC	Primer	For 5' or 3' RACE
gsp2_prom2128	GCATCGGAAATACGGATCAT	Primer	For 5' or 3' RACE
gsp2_plus58	ATCGCGAATATCCAGCAATC	Primer	For 5' or 3' RACE
gsp2_plus67	AACACGACCACGAACACAAA	Primer	For 5' or 3' RACE
gsp2_plus69	CACTTTCACGTCCATCACCA	Primer	For 5' or 3' RACE
gsp2_rprA	TCAGGGGATTTCCATGCTTA	Primer	For 5' or 3' RACE
gsp2_gcvB	CAGAACACGCATTCCGATAA	Primer	For 5' or 3' RACE
gsp2_tp2	TTGCTCGACAGAAGTCGTGA	Primer	For 5' or 3' RACE
gsp2_gadY	AGGATAGTCTGCCGTCTCCA	Primer	For 5' or 3' RACE
gsp2_fimA3prime	GCGTTAACAACTTCCCCTTT	Primer	For 5' or 3' RACE
gsp2_plus19	GTAATAGTTTCGGGCGCTTC	Primer	For 5' or 3' RACE
gsp2_plus22	TCGTTCGTTTTACCCATTGTC	Primer	For 5' or 3' RACE
gsp2_plus45	CCAACTTCACCCTGGCAACC	Primer	For 5' or 3' RACE
gsp2_minus53	GCGTCCATCAAAGTACTGGAG	Primer	For 5' or 3' RACE
ORACEF1	GAGGACTCGAGCTCAAGC	Primer	For 5' or 3' RACE
4_7_reg26RT_L	CCGATGCGTTATCGTAATCA	Primer	qRT-PCR
4_7_reg26RT_R	AGCATCATATTGGGCGACAT	Primer	qRT-PCR
RT_cutC_L	ATGCCACGAATGGAAAAA	Primer	qRT-PCR
RT_cutC_R	CGTCTGATTTTTGCCCTGAT	Primer	qRT-PCR

MATERIALS AND METHODS

Growth conditions, RNA extraction, and cDNA preparation for qRT-PCR or microarrays

Cultures of *E. coli* were established as previously described (4) and growth in either LB or M9 complete minimal media with 0.2% glucose or 0.2% maltose, as indicated. When appropriate antibiotics were applied at the following concentrations: 100 ug/ml ampicillin, 20 ug/ml chloramphenicol. Culture samples were taken immediately prior to induction (time 0, t0), and 20 minutes after induction (time 20, t20). Exponential phase induction indicates an OD_{450} = 0.3, and stationary phase induction indicates an OD_{450} = 1.2 just prior to induction with IPTG. 8 ml samples were removed, and added to ice-cold 5% water-saturated phenol in ethanol solution, centrifuged at 6,600 g for 2 minutes, and the cell pellets flash-frozen in liquid nitrogen before storing at -80°C until required for RNA preparation using the hot phenol technique (78) followed by cDNA preparation (79).

RNA was extracted using the hot phenol technique (78), with the following modifications. Briefly, cell pellets were resuspended with 500 µl of lysis solution (320 mM Na acetate at pH 4.6, 8% SDS, 16 mM EDTA), and mixed at 65°C for 10 minutes with 1 ml of 65°C water-buffered phenol. The samples were then placed on ice for 5 minutes, then centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was extracted by phenol-chloroform twice, precipitated with 2.5 vol of 100% ethanol, and the resulting RNA pellet resuspended in 85 µl of RNase free water. Genomic DNA was then removed from the samples using Turbo DNA-free Turbo Dnase Treatment according to the manufacturer's directions for rigorous DNase treatment (Applied Biosystems, Foster

City, CA, USA). cDNA was prepared for qRT-PCR as previously described using 5 μg of input RNA (79). A minimum of three independent experiments was performed for each strain and condition.

Probe preparation, procedure, and microarray analysis

mRNA transcripts present at significantly different levels in strains before and after sRNA or RpoE overexpression in a given condition were determined by hybridizing fluorescently labeled cDNA to custom Nimblegen *E. coli* microarrays (courtesy of Dr. Robert Landick Madison, WI). Targets were considered significant if they were regulated more than two-fold when compared to before sRNA or RpoE overexpression in any given condition.

Cy3 and Cy5 cDNA was prepared from 10 μg of total RNA with 16 μg of random hexamer as described in (4). Relative mRNA levels were determined by parallel two-color hybridizations to custom Nimblegen *E. coli* microarrays (courtesy of Dr. Robert Landick Madison, WI) that contains two copies of 187,204 T_m-matched ≥45-mer oligonucleotides that tile the *E. coli* chromosome with an average of spacing of 24.5 bp. Probe intensities were summarized to generate expression values for each ORF using RMA normalization as described in the NimbleScan User's Guide and intensity(dye)-dependent biases corrected for using lowess smoothing from MA plots. mRNA transcripts present at significantly different levels in strains before and after sRNA overexpression in a given condition were determined by hierarchical clustering using the software Cluster and visualized by using Treeview (http://rana.lbl.gov/EisenSoftware.htm) (80).

Analysis of translational control and target recognition using target-*gfp* fusion plasmids

E. coli strains harboring *gfp* fusion (*gfp* alone, or target-*gfp* fusions) and sRNA expression plasmids were grown overnight to saturation and fluorescence values measured using a multimode microplate reader-incubator shaker Varioskan (excitation = 481 nm, emission = 507 nm, Thermo Fisher Scientific). "-" indicates strain has parent (control) plasmid that does not express sRNA; "WT" , "M2", etc., indicate the sRNA variant expressed by the plasmid. For fold change calculations, GFP fluorescence of strain expressing only the target-GFP fusion is set at 1. Fold-change indicates ratio of GFP fluorescence of (strain expressing both sRNA and target / strain expressing target only). The average of three experiments with SD is shown.

Gene expression analysis using qRT-PCR or by β-galactosidase assay

qRT-PCR reactions were carried out using Stratagene Brilliant II Sybrgreen master mix according to the manufacturer's directions (Agilent Technologies, La Jolla, CA, USA), and 6 pmol each forward and reverse primers (Integrated DNA Technologies; see Supplementary Table 3). Real-time PCR was performed with a Stratagene Mx3000P sequence detection system (Agilent Technologies). Data were analyzed using the method described in (81) with *recA* and *gyrA* as internal control genes, and the transcript abundance of each mRNA was quantified relative to time=0 of its own genotype and are

plotted as $\log_2$ fold change. A minimum of three independent experiments were performed for each strain and condition.

Northern Analysis

For detection of the sRNAs, total RNAs (10 µg) were fractionated in 8% polyacrylamide-8 M urea gels and transferred to a BrightStar Positively Charged Nylon membrane (Ambion, Austin, Texas). Membranes were hybridized with biotinylated oligo probes (Supplementary Table 3) overnight in Ultrahyb Oligo buffer (Ambion, Austin, Texas) at 42°C and subsequently washed according to the BrightStar BioDetect Protocol (Ambion, Austin, Texas).

Whole cell protein fractions and Western blot

E. coli TOP10 F' cells were transformed with the RybB/RybB* expression plasmids (or control plasmid pJV300) and the *gfp*-reporter plasmids. Co-transformants were grown to OD_{600}=0.1, sRNA expression was induced by addition of 1mM IPTG (final concentration.) and cultivation was continued until cells reached OD_{600}=1.0. Culture samples were taken according to OD_{600} and centrifuged 2 min at 16,100 g at 4°C. The cell pellet was resuspended in 1x sample loading buffer (1X SLB; Fermentas) to a final concentration of 0.01 OD/ml. Western Blotting and detection of GFP fusion proteins and GroEL was performed as described previously (82).

Plasmid Construction

The plasmid CAG62157 is a derivative of the plasmid CAG25196, and was constructed by annealing oligos ptrcEG_operator top and ptrcEG_operator bottom, performing a partial SspI, MscI digest on CAG25196, and ligating in the annealed oligos. The end result is an IPTG inducible Trc promoter that allows for MscI cloning of expression constructs. When MscI cloned constructs are expressed, the resulting transcript will not contain any additional nucleotides from the plasmid sequence. Constitutively expressed, in-frame target-GFP fusion plasmids derived from pXG-10 were constructed as described in (83). Derivatives of the target-GFP fusion plasmids harboring point mutations were generated using QuickChange mutagenesis according to the manufacturer's directions (Agilent Technologies, La Jolla, CA, USA). Construction of Pfiu::gfp (pKP-192-1) and PrluD::gfp (pKP-210-1) reporter plasmids was achieved by amplification of E. coli K12 DNA fragments spanning from -117 to 60 bps and -54 to 60 bps (corresponding to the *fiu* or *rluD* translational start sites) of the *fiu* or *rluD* coding sequence using oligonucleotides JVO-4798/-4799 and JVO-4796/5557, respectively. The PCR products were digested with BrfBI and NheI, gel-purified and ligated into the pXG-10 plasmid (84) digested with the same enzymes. These plasmids served as templates for establishment of pfiu*::gfp (pKP-209-1) and prluD*::gfp (pKP-218-3) harbouring a single nucleotide exchange which was introduced by primers JVO-5301/5302 and JVO-5656/5657. Competent E. coli TOP10 or TOP10 F' cells (Invitrogen) were used for all cloning procedures. Control-plasmid pJV300 (85) as well as RybB-expression plasmids pFM-1-1 (wild-type RybB) and pFM-17-2 (RybB*) were previously published (9).

Promoter Strength and Mapping

In vivo promoter strength was carried out as described previously (3). The Reg26 promoter reporter contains the region from -65 through +5 driving expression of *gfp*. 5' and 3' RACE was carried out as described previously (4, 63).

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