MicL, a new $\sigma^E$-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein

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In enteric bacteria, the transcription factor $\sigma^E$ maintains membrane homeostasis by inducing synthesis of proteins involved in membrane repair and two small regulatory RNAs [sRNAs] that down-regulate synthesis of abundant membrane porins. Here, we describe the discovery of a third $\sigma^E$-dependent sRNA, MicL [mRNA-interfering complementary RNA regulator of Lpp], transcribed from a promoter located within the coding sequence of the cutC gene. MicL is synthesized as a 308-nucleotide [nt] primary transcript that is processed to an 80-nt form. Both forms possess features typical of Hfq-binding sRNAs but surprisingly target only a single mRNA, which encodes the outer membrane lipoprotein Lpp, the most abundant protein of the cell. We show that the copper sensitivity phenotype previously ascribed to inactivation of the cutC gene is actually derived from the loss of Micl and elevated Lpp levels. This observation raises the possibility that other phenotypes currently attributed to protein defects are due to deficiencies in unappreciated regulatory RNAs. We also report that $\sigma^E$ activity is sensitive to Lpp abundance and that Micl and Lpp comprise a new $\sigma^E$ regulatory loop that opposes membrane stress. Together MicA, RybB, and MicL allow $\sigma^E$ to repress the synthesis of all abundant outer membrane proteins in response to stress.

[Keywords: sRNA; Hfq; cutC; copper; outer membrane homeostasis; $\sigma^E$]

Supplemental material is available for this article.

Received April 13, 2014; revised version accepted June 17, 2014.

The outer membrane (OM) of Gram-negative bacteria is its first line of defense against the environment, as it is a barrier against antibiotics and other stresses (for review, see Nikaido 2003). The OM is a complex environment consisting of outer leaflet lipopolysaccharide (LPS), inner leaflet phospholipids, and proteins such as OM porins (OMP) and lipoproteins (for review, see Narita and Tokuda 2010; Silhavy et al. 2010; Ricci and Silhavy 2012; Zhang et al. 2013). The major Escherichia coli lipoprotein Lpp resides in the OM and is the most abundant protein in the cell (~1 million copies), comprising 2% of its dry weight (Narita and Tokuda 2010; Li et al. 2014). Approximately a third of the Lpp pool is conjugated to the peptidoglycan layer, serving as a structural element that connects the OM to the peptidoglycan (Braun and Rehn 1969; Inouye et al. 1972), while the remainder exists, at least in part, as a surface-exposed form that can be recognized by antimicrobial peptides (Cowles et al. 2011; Chang et al. 2012). Since cells synthesize a new OM each cell cycle, OM components are synthesized and transported at a tremendous rate. Indeed, at 37°C, >5% of all active ribosomes are devoted to Lpp translation (Li et al. 2014). Therefore, balancing the massive flux of membrane components with sufficient transport and assembly factors is vital for OM homeostasis.

In E. coli and related $\gamma$-proteobacteria, OM homeostasis is monitored by the essential transcription factor $\sigma^E$, which responds to perturbations to OMP and LPS folding...
MicL for mRNA-interfering complementary RNA regulator of Lpp, following the nomenclature of Mizuno et al. [1984]. MicL is transcribed from a strong $\sigma^E$-dependent promoter within the cutC coding sequence and subsequently processed into a smaller transcript [MicL-S]. It is responsible for all phenotypes previously associated with loss of cutC. We discuss how our finding that MicL/Lpp constitute a novel regulatory loop modulating $\sigma^E$ activity expands our view of the cellular mechanism for maintaining OM homeostasis as well as the implications of sRNAs evolving from the 3' end of transcripts.

Results

$\text{MicL is a third } \sigma^E\text{-regulated sRNA}$

To identify novel $\sigma^E$-dependent sRNAs in *E. coli*, we used a tiled microarray to examine whole-genome expression after ectopic $\sigma^E$ overexpression. Along with the previously identified $\sigma^E$-dependent sRNAs MicA and RybB, we observed two overlapping transcripts that were strongly up-regulated in a $\sigma^E$-dependent manner within the 3' end of cutC and the intergenic region between cutC and torY [Fig. 1A]. These transcripts are likely the same as RyeF, a putative sRNA previously identified in the cutC/torY intergenic region of *E. coli* and *Salmonella* (Zhang et al. 2003a; Chao et al. 2012). We did not observe a $\sigma^E$-dependent transcript upstream of cutC, suggesting that cutC itself is not $\sigma^E$-dependent (data not shown). Additionally, we did not observe the previously postulated $\sigma^E$ regulation of CyaR [Johansen et al. 2008], suggesting that this sRNA is unlikely to be directly regulated by $\sigma^E$ (data not shown).

Northern analysis of total RNA isolated from cells with and without ectopic expression of $\sigma^E$ validated the presence of two $\sigma^E$-dependent transcripts, an ~300-nucleotide (nt) transcript denoted as MicL and an ~80-nt transcript denoted as MicL-S, which were detected with a probe to the 3' end of cutC [Fig. 1B]. Both MicL and MicL-S are induced during transition to stationary phase, a time when $\sigma^E$ activity increases dramatically [Ades et al. 1999; Costanzo and Ades 2006]. The two bands showed maximal expression during late stationary phase in defined rich medium (around ~15 h) [Fig. 1C] and in LB [data not shown], consistent with $\sigma^E$ induction.

Primer extension and total mRNA sequencing [mRNA-seq] analysis revealed that the 308-nt MicL transcript begins within cutC [226 nt before the cutC stop codon] and ends at the cutC intrinsic terminator, significantly upstream of the start of torY [Supplemental Fig. S1A,B, data not shown]. The 80-nt MicL-S begins with the last base of the cutC stop codon and ends at the cutC terminator. Thus, both forms of MicL contain the full cutC 3' untranslated region [UTR].

We identified a putative $\sigma^E$ promoter upstream of the start of MicL [P$_{micL}$] [Fig. 1D; Rhodius et al. 2006] but not in front of MicL-S. Strong conservation of this sequence within the cutC coding sequence is observed in *Shigella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Cronobacter*, and...
Enterobacter species but not in more distantly related enteric bacteria (Supplemental Fig. S2A–D). A fusion of the minimal putative P\text{micL} promoter (−65 to +20) to GFP is induced by ectopic s\text{E} overexpression and is only slightly weaker than the strong s\text{E}-dependent micA and rybB promoters in the same vector background (Fig. 1E). Together, these data show that MicL is a third s\text{E}-dependent sRNA in \textit{E. coli} and likely in related enteric bacteria.

**MicL-S is processed from MicL**

MicL-S may be processed from MicL, as we did not observe a promoter for MicL-S. We tested this by treating total RNA with 5’ monophosphate-dependent terminator exonuclease (TEX), which degrades processed transcripts but spares primary transcripts, as they have 5’ triphosphates. Following TEX treatment, MicL-S is degraded, but the MicL level is virtually unchanged (Fig. 1F), suggesting that MicL-S is generated by ribonucleolytic cleavage of MicL.

We examined MicL levels after 15 min of MicL induction from P\text{lacO-1} and subsequent IPTG washout (Supplemental Fig. S3A). The observations that MicL-S is detected only after induction of MicL and that MicL and MicL-S disappear with similar kinetics support the idea that MicL-S is derived from MicL. Importantly, MicL-S expressed independently from the P\text{lacO-1} promoter has the same half-life as MicL-S cleaved from MicL (Supplemental Fig. S3B), demonstrating that cleavage does not impact MicL-S stability.

We next investigated the mechanism of MicL processing. Although the MicL cleavage site is within the cutC TGA stop codon, this sequence is not a cleavage signal, as a TGA-to-GGA mutation did not alter processing (Supplemental Fig. S3C). RNase E is the primary RNase in \textit{E. coli} and mediates processing of other sRNAs (Massé et al.
but production of MicL-S was not abolished in a rne-3071 mutant (Supplemental Fig. S3D) or in strains lacking various other RNases (Opdyke et al. 2011), including RNase III (rne), RNase G (rng), RNase BN (elaC), five toxin endonucleases (Supplemental Fig. S3E), and the broadly conserved YbeY RNase (data not shown). Either uncharacterized ribonucleases mediate MicL processing or other combinations of RNases perform this function.

_Lpp is the sole target of MicL_

Transcripts from the 3’ UTR of cutC [RyeF] coimmunoprecipitate with Hfq in _E. coli_ and _Salmonella_ (Zhang et al. 2003a; Chao et al. 2012). We validated this observation for both MicL and MicL-S, which coimmunoprecipitate with Hfq at ratios consistent with their levels, suggesting that both forms bind Hfq with similar affinity (Fig. 2A). In addition, both MicL transcripts are virtually undetectable in strains lacking Hfq (hfq-1), indicating that their stabilities are Hfq-dependent (Fig. 2A).

Hfq-binding sRNAs in _E. coli_ have all been found to regulate target mRNAs via limited base-pairing, enabling them to regulate expression of multiple targets. With this expectation, we searched for targets of MicL. However, analysis of mRNA-seq data taken before and after expression of MicL for 4, 10, and 20 min identified only a single MicL target, _lpp_ (Fig. 2B; Supplemental Fig. S4A; Supplemental Table S1). The levels of _lpp_ mRNA were reduced starting 4 min after induction and were down-regulated by 20-fold after 20 min (Supplemental Fig. S4C). The OM lipoprotein Lpp, the most abundant protein in the cell, is a key component of the membrane. α^5^ was previously reported to repress _lpp_ via an unknown mechanism that required Hfq (Rhodius et al. 2006; Guisbert et al. 2007). Stunningly, even after the 20-fold reduction in _lpp_ mRNA due to MicL overexpression, _lpp_ is still the 12th most abundant mRNA in the cell (Supplemental Table S1).

We examined the possibility that other MicL targets might be regulated solely at the level of translation by sequencing ribosome-protected mRNA fragments [ribosome profiling] (Ingolia et al. 2009) after ectopic expression of MicL at the same time points used above for mRNA-seq (Fig. 2C; Supplemental Table S2). Similar to what we observed for the steady-state mRNA levels, expression of MicL decreased translation of _lpp_ ~10-fold after a 20-min induction of MicL. For all other transcripts, translation was not significantly altered by MicL overexpression (Supplemental Fig. S4E,F). _lpp_ is the most well-translated mRNA in the cell and remains the 30th most well-translated mRNA after MicL expression (Supplemental Table S2). Together, these experiments strongly suggest that _lpp_ is the sole MicL target under the conditions tested.

_MicL repression of Lpp mimics lpp deletion phenotypes_

Strains lacking Lpp were reported to be sensitive to membrane perturbants such as dibucaine, deoxycholate, sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA) (Hirota et al. 1977; Suzuki et al. 1978; Nichols et al. 2011). Using the reported concentrations of 2003), but production of MicL-S was not abolished in a rne-3071 mutant (Supplemental Fig. S3D) or in strains lacking various other RNases (Opdyke et al. 2011), including RNase III (rne), RNase G (rng), RNase BN (elaC), five toxin endonucleases (Supplemental Fig. S3E), and the broadly conserved YbeY RNase (data not shown). Either uncharacterized ribonucleases mediate MicL processing or other combinations of RNases perform this function.
for these chemicals, we found that dibucaine yielded the strongest distinction between wild-type and Δlpp strains, with the latter having small, translucent colonies in the presence of dibucaine. [Fig. 3A]. Cells harboring MicL or MicL-S appeared mildly translucent on dibucaine in the absence of inducer and become markedly translucent after addition of inducer [cf. Fig. 3A and Supplemental Fig. S5]. Δlpp cells additionally display a small (~10-fold) decrease in viability, but this was not observed for wild-type cells overexpressing either MicL or MicL-S, possibly because such cells still retain some Lpp. Overexpression of MicL or MicL-S in a Δlpp background did not further sensitize cells to dibucaine [Fig. 3A], supporting the conclusion that the dibucaine sensitivity associated with MicL overexpression is due to decreased lpp levels.

Endogenous levels of MicL are sufficient to repress lpp

To determine whether MicL expressed from its native locus had the capacity to repress lpp, we assayed lpp mRNA levels upon σE overexpression. Indeed, elevated σE led to reduced lpp mRNA in wild-type cells but not in a strain lacking MicL (ΔcutC) [Fig. 3B]. We also tested whether lpp mRNA was down-regulated in stationary phase when MicL levels are highest [Fig. 1C]. As can be seen in Figure 3C, in stationary phase [10 or 15 h of growth], lpp transcript levels are less abundant in wild-type cells than in cells lacking MicL. We also observed higher accumulation of Lpp protein in the ΔcutC strain compared with the wild-type strain. The Lpp protein level does not mirror changes in lpp mRNA, as the protein is stable and therefore accumulates in stationary phase because proteins are no longer diluted by cell division. Interestingly, even in the ΔcutC strain, we saw a sharp decrease in lpp mRNA levels during stationary phase, suggesting the existence of additional regulators of lpp expression and highlighting the importance of reducing Lpp levels in stationary phase [Fig. 3C].

MicL-S base-pairs directly with lpp mRNA

To test for direct base-pairing between MicL and lpp, we generated a translational fusion by integrating the lpp 5' UTR (containing sequences from the transcription start site through 102 nt of the lpp coding sequence) in-frame to the seventh codon of lacZ gene, all downstream from the heterologous PBAD promoter in the chromosome of PM1205 [Mandin and Gottesman 2009]. The β-galactosidase activity of this reporter strain was reduced more than twofold by ectopic overexpression of both MicL

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**Figure 3.** MicL repression of lpp is physiologically important. [A] Expression of MicL phenocopies the dibucaine sensitivity of Δlpp. Wild-type or Δlpp cells carrying pBR*-MicL, pBR*-MicL-S, or empty vector were spotted at the indicated dilutions on LB plates containing 1.4 mM dibucaine with or without 1 mM IPTG. [B] MicL represses lpp RNA levels following σE overexpression. Wild type and a ΔcutC strain with either control vector or pRpoE growing exponentially in LB (OD600 ~0.1) were induced with 1 mM IPTG for 2 h. Total RNA was isolated and probed for lpp, MicL, and 5S RNA. [C] lpp mRNA and Lpp protein levels in wild-type and ΔcutC mutant backgrounds. At the indicated times, total RNA was extracted from wild type and the ΔcutC mutant strain grown in LB. Total RNA was probed to examine lpp, MicL, and 5S RNA levels, and Lpp and GroEL protein levels were examined by immunoblotting protein samples taken at the same time points. For B and C, the intensity of the lpp RNA or protein band for each strain was quantified using ImageJ software, and the ratios between the corresponding samples for the ΔcutC mutant and wild-type strains are given.
and MicL-S but not by overexpression of MicA or RybB (Fig. 4A). As both forms of MicL down-regulate lpp, the region required for regulation must be within MicL-S. To further define the regulatory sequences, we tested whether 5’ truncations of MicL-S retained the ability to regulate the lpp-lacZ translational reporter. A MicL-S variant lacking the first 12 nt [MicL-SA1] fully repressed the fusion, while a MicL-S variant lacking the first 45 nt [MicL-SA2] did not, placing the sequence required for regulation between nucleotides +13 and 44 of MicL-S (Supplemental Fig. S6). We similarly defined the MicL-responsive region of lpp, finding that a truncation retaining the first 33 nt of the lpp coding sequence is repressed by MicL-S [lppA2], but a truncation that retains only the first 6 nt of the coding sequence is not [lppA3], suggesting that a portion of the sequence targeted by MicL lies between +6 and 33 nt of the lpp coding sequence [+43–70 nt from the start of the of lpp mRNA] (Supplemental Fig. S6).

Computational analysis of these regions using ThermoComposition software (Matveeva et al. 2007) also suggested possible base-pairing between +19 and 49 of MicL-S [Fig. 4B,C] and +16 and 46 of the lpp coding sequence. Indeed, MicL-S-1, harboring a 4-nt mutation in the predicted pairing region of MicL-S [altered nucleotides +41–44] [Fig. 4B,C], was unable to repress the lpp-lacZ reporter [Fig. 4D], but a compensatory mutation in lpp [lpp-lacZ-1, altered nucleotides +21–25 of the coding sequence] restored repression to levels comparable with wild-type regulation [Fig. 4D]. We verified that MicL-S and MicL-S-1 accumulate to similar levels, and while MicL-S noticeably reduced lpp mRNA and Lpp protein levels, MicL-S-1 does not [Fig. 4E]. Thus, MicL-S is an sRNA that directly base-pairs with and represses lpp.

Stable duplex predictions between cutC and lpp in various bacteria revealed that the extensive region of base-pairing—and particularly a stable core (seed) interaction between +38 and 49 of MicL-S and +16 and 28 of the lpp coding sequence—is conserved in only a select group of enteric bacteria, consistent with a recent evolution of the MicL RNA (Supplemental Figs. S2A–D, S7). Interestingly, while Salmonella enterica contains two lpp genes [lppA and lppB], the long stretch of MicL complementarity is detected for only one of the two lpp genes [lppA] found in this organism.

**MicL represses lpp by inhibiting translation**

Most sRNAs inhibit translation by sterically occluding the Shine-Dalgarno sequence or the start codon, prevent-
ing ribosomes from accessing target mRNA [for review, see Desnoyers et al. 2013]. As the core of Micl base-pairing with lpp is downstream from the translation start site (+16–28 nt of the lpp coding sequence) [Fig. 5A], at the edge of the region where sRNA binding is known to interfere with translation initiation [Bouveri et al. 2008], it is unclear whether Micl represses translation or affects mRNA stability independently of translation. To examine this, we tested whether Micl-S overexpression reduces the mRNA levels of lpp derivatives harboring early stop mutants [stop codon at the start and second and fourth codons] [Fig. 5A]. While the lpp stop mutant at the start codon cannot be translated, translation should initiate for the other two derivatives. Although the absolute levels of lpp mRNA are altered, we no longer observed a significant decrease in lpp mRNA levels following Micl-S overexpression in any of these strains [Fig. 5B]. This suggests that the primary effect of Micl is to inhibit translation of lpp rather than to mediate lpp mRNA degradation and that increased degradation is a consequence of the fact that untranslated mRNAs are not protected from ribonucleolytic cleavage [Nilsson et al. 1984].

Consistent with the idea that lpp mRNA is rapidly degraded in the absence of active translation, we observed that expression of Micl did not significantly decrease the translation efficiency [ribosomes per unit mRNA] of lpp [Fig. 5C]. This suggests that every lpp mRNA is being translated by the same number of ribosomes regardless of the level of Micl. Thus, lpp mRNA either is undergoing active translation or is rapidly cleared when Micl binding blocks translation.

Phenotypes ascribed to ∆cutC are due to eliminating Micl repression of lpp

The cutC gene was reported to be involved in copper homeostasis because missense mutations in cutC alone and in combination with mutations in nlpE lead to copper sensitivity [Gupta et al. 1995]. Interestingly, the cutC mutations leading to copper sensitivity are clustered around the Pmicl promoter: One lies between the Pmicl−10 and −35 motifs [nucleotide change G197A, amino acid change R66H], and the other is located at −67 from the Pmicl start [nucleotide change A146G, amino acid change K49R], raising the possibility that the copper phenotype of cutC could be due to misregulation of Micl. We tested this possibility by determining the copper phenotype of two constructs: a 5′ deletion of cutC that maintains Micl but deletes the first 104 codons of cutC (cutCΔ5′) and a Micl promoter mutant [point mutations in Pmicl−10 and −35 motifs] that conserves CutC protein sequence [Pmicl mutant]. Northern analysis confirmed that Micl and Micl-S expression was nearly abolished by Pmicl mutation [Supplemental Fig. S8B], and Western analysis confirmed that CutC is not synthesized in the cutCΔ5′ mutant [Supplemental Fig. S1C]. The Micl levels were moderately reduced in cutCΔ5′ cells, possibly due to effects on Pmicl [Supplemental Fig. S8B]. However, only the Pmicl mutant has a copper sensitivity phenotype that closely matches that of ∆cutC [Fig. 6A; Supplemental

Figure 5. Micl repression of lpp is dependent on translation. |A| Diagrammatic representation of the derivatives carrying early stop codon mutations lpp-1 [ATG to TAG at the first codon], lpp-2 [AAA to TAA at the second codon], and lpp-4 [ACT to TAA at the fourth codon]. |B| The pBR*-Micl-S plasmid was transformed into wild-type and lpp translation-defective cells, Micl-S was induced with 1 mM IPTG in LB for 3 h, and RNA was extracted (final OD600 ~ 1.0) and probed for lpp, Micl-S, and SS RNA. The intensity of the lpp band from each strain was quantified using ImageJ software, and the fold changes listed below are calculated for the corresponding samples with and without IPTG. Immunoblot analysis for Lpp confirmed that translation was eliminated in the stop codon mutants [data not shown]. |C| Translation efficiency of Lpp is unchanged after Micl expression. Translation efficiency per gene after 20 min of Micl induction is plotted versus translation efficiency before Micl induction. Translation efficiency was calculated as the number of ribosome footprints per gene/mRNA reads per gene from the ribosome profiling and mRNA-seq data.

Fig. S9]. Furthermore, ectopic expression of either Micl or Micl-S dramatically increased the viability of ∆cutC on copper [Fig. 6B] without affecting growth [Supplemental Fig. S8D]. Micl overexpression also enhanced copper resistance in wild-type cells [Fig. 6B]. As lpp is the sole target of Micl, we tested whether reduced synthesis of Lpp underlies copper resistance. Indeed, a ∆lpp strain was slightly more resistant to copper than wild-type cells [Fig. 6A, Supplemental Fig. S9], and overexpression of Micl and Micl-S did not increase the copper resistance of ∆lpp mutants [Fig. 6B]. Together, these
and with Lpp accumulation. We tested whether the MicL, independent regulatory loop that opposes stresses associated in a previously described regulatory loop (Papenfort et al. 2010; Pen˜as et al. 1997; Hayden and Ades 2008), as was observed for MicA and RybB overexpression (Papenfort et al. 2010; Gogol et al. 2011). The ~50-fold to 100-fold decrease in viability caused by overexpressing the $\sigma^E$ negative regulators RseA and RseB is rescued comparably by coexpressing either MicL or MicA (Fig. 7C; Supplemental Fig. S10D). We conclude that MicL and Lpp represent an additional sRNA loop with an OM-protective function similar to the other $\sigma^E$-dependent sRNAs.

**Figure 6.** Copper sensitivity of $\Delta cutC$ is due to loss of MicL. (A) Sensitivity of wild-type strains and variants with $P_{micL}$ mutant (-10C-T/-35A-G), cutCΔ5 (which preserves MicL), $\Delta cutC$, and $\Delta lpp$ to 4 mM Cu(II)Cl2. Three microliters of each strain in exponential phase was spotted on LB supplemented with 4 mM Cu(II)Cl2 at the indicated dilutions (Tetaz and Luke 1983; Gupta et al. 1995). (B) Sensitivity of wild-type cells, $\Delta cutC$, and $\Delta lpp$ transformed with pBR*- control vector, pBR*-MicL-S, and pBR*- MicL to 4 mM Cu(II)Cl2 using conditions in A with the exception that the medium was additionally supplemented with kanamycin. Some differences in sensitivity between A and B may be due to a synthetic effect between copper and the kanamycin used for plasmid selection in B.

Data suggest that high levels of Lpp result in copper sensitivity and that MicL confers copper resistance by reducing Lpp levels.

$\sigma^E$, MicL, and Lpp form a protective regulatory loop

The essential transcription factor $\sigma^E$ regulates the folding and levels of abundant membrane proteins such as OMPs. In a previously described regulatory loop (Papenfort et al. 2010; Gogol et al. 2011), $\sigma^E$ is activated by unfolded OMPs and in turn induces expression of the MicA and RybB sRNAs, which oppose stress by down-regulating OMP mRNAs. Micl and Lpp may constitute another $\sigma^E$-dependent regulatory loop that opposes stresses associated with Lpp accumulation. We tested whether the MicL, Lpp, and $\sigma^E$ relationship was similar to that established for RybB and MicA, OMPs, and $\sigma^E$. Indeed, $\sigma^E$ activity responds to Lpp levels. Although Lpp is already the most abundant protein in the cell, mild overexpression of Lpp (approximately twofold) leads to activation of the $\sigma^E$ response, and high overexpression (approximately threefold) leads to significant $\sigma^E$ activity and growth arrest (Fig. 7A; Supplemental Fig. S10A).

Others have found that $\sigma^E$ activity is inhibited in cells that have lost lpp (Mecas et al. 1993). Similarly, we observed that reducing Lpp levels 10-fold by MicL overexpression leads to a reduction in $\sigma^E$ activity (Fig. 7B; Supplemental S10B). In addition, Northern analysis showed that cells lacking MicL ($\Delta cutC$ strain) have ~1.5-fold higher RybB levels in stationary phase (Supplemental Fig. S10C), consistent with higher $\sigma^E$ activity.

Finally and most importantly, overexpression of MicL is able to rescue the growth defect associated with depletion of $\sigma^E$ activity (Fig. 7C; Supplemental Fig. S10D; De Las Peñas et al. 1997; Hayden and Ades 2008), as was observed for MicA and RybB overexpression (Papenfort et al. 2010; Gogol et al. 2011). The ~50-fold to 100-fold decrease in viability caused by overexpressing the $\sigma^E$ negative regulators RseA and RseB is rescued comparably by coexpressing either MicL or MicA (Fig. 7C; Supplemental Fig. S10D). We conclude that MicL and Lpp represent an additional sRNA loop with an OM-protective function similar to the other $\sigma^E$-dependent sRNAs.

**Discussion**

Lpp is the most abundant protein in the cell and is of central importance in OM homeostasis. It is both embedded in the OM and covalently linked to the peptidoglycan layer, forming an important linkage that connects the OM to the rest of the cell. In this study, we established that MicL, a $\sigma^E$-dependent sRNA, specifically targets lpp mRNA, preventing its translation. We show that lpp is the sole MicL target under conditions that we tested. This stands in contrast to most sRNAs, which act via limited base-pairing to regulate multiple targets. Additionally, MicL is transcribed from within the coding region of the gene cutC, and we show that it is responsible for all known phenotypes of cutC. Our results put $\sigma^E$ at the center of an sRNA and protein network that monitors lipoprotein biogenesis and regulates the majority of proteins destined for the membrane.

**MicL is a dedicated regulator of Lpp**

Lpp exists in ~1 million copies per cell (~2% of dry cell weight) (Narita and Tokuda 2010; Li et al. 2014) and comprises ~10% of all cellular mRNA and ~8% of all translation events in our conditions. Loss of Lpp leads to a weakened and less tethered OM, causing increased vesiculation, leakage of periplasmic contents, and sensitivity to a variety of compounds (Hirota et al. 1977; Suzuki et al. 1978). Inappropriate up-regulation of Lpp likewise is deleterious: Defects in Lpp transport or mislocalization of Lpp to the inner membrane leads to cell death (Yakushi et al. 1997). Thus, the levels of this protein must be maintained in a narrow range for optimum growth.

Two unique features of the Lpp life cycle make post-transcriptional regulation by MicL attractive. First, the cell cannot respond to defects in Lpp transport by up-regulating lipoprotein chaperones and transport machines, as these factors use some of the same transport machines
as Lpp [Narita and Tokuda 2010]. Second, transcriptional repression will not rapidly lower Lpp flux, since lpp mRNA is unusually stable ($T_{1/2} \sim 10$ min in vivo) [Nilsson et al. 1984; Ingle and Kushner 1996]. MicL repression of Lpp translation elegantly solves both problems: Blocking ribosome initiation on lpp decreases Lpp translation and accelerates degradation of lpp mRNA to < 4 min based on analysis of our mRNA-seq data. Increased degradation is likely the result of both increased access to RNases, resulting from decreased translation, and recruitment of RNase E through its association with Hfq. MicL-mediated regulation has a further advantage because sRNAs continually inhibit their targets. This is likely to generate less variance in mRNA expression than inhibition of transcription (Levine et al. 2007), which can generate bursts in mRNA synthesis when repressors transiently dissociate from DNA [for review, see Eldar and Elowitz 2010].

It is notable that MicL has only a single mRNA target. This stands in contrast to all other Hfq-binding sRNA regulators characterized thus far. Lpp might necessitate an sRNA dedicated to controlling the rate of its synthesis due to its enormous abundance. Since lpp is in such high excess over other mRNAs, a second target may be difficult to regulate, as competition for base-pairing with MicL could prevent the down-regulation of the less well-expressed transcript [Levine et al. 2007] such that the secondary mRNA targets would not be regulated until most of the lpp mRNA is degraded.

$s^\varepsilon$-Regulated sRNAs repress protein synthesis of all of the most abundant OM proteins

Our results place $s^\varepsilon$ at the center of an elaborate regulatory system that monitors and responds to defects in all aspects of the OM biogenesis [Fig. 8]. $s^\varepsilon$ senses OM status through the degradation rate of its negative regulator, RseA, which is mediated by DegS and RseB. DegS and RseB respond, respectively, to misaccumulation of OMPs and LPS. Upon stress, $s^\varepsilon$ up-regulates proteins facilitating OMP and LPS assembly and transport. In addition, $s^\varepsilon$ up-regulates the MicA and Ryb8 sRNAs to down-regulate OMP synthesis and, as we showed here, MicL to down-regulate Lpp synthesis. The MicA and Ryb8 sRNAs are part of a regulatory loop that opposes stresses associated with OMP folding and assembly. Our data for MicL/Lpp indicate that they constitute a second $s^\varepsilon$-dependent protective regulatory loop to oppose stresses associated with Lpp folding. We suggest that $s^\varepsilon$ senses Lpp status as an indirect consequence of monitoring OMP and LPS assembly. The essential lipoprotein components of the OM assembly machines of OMPs [BamD] and LPS [LptE] [for review, see Silhavy et al. 2010] are in direct competition with Lpp, as all lipopro-

Figure 7. MicL and Lpp are part of an envelope protective regulatory loop. (A) Overexpression of Lpp increases $s^\varepsilon$ activity. Cells with either control vector or pTrc-Lpp were induced with either 50 $\mu$M or 1 mM IPTG [at the time indicated]; $s^\varepsilon$ activity was measured from a $s^\varepsilon$-dependent rpoHp3-lacZ reporter. The $s^\varepsilon$ activity for the vector control strain treated with 50 $\mu$M or 1 mM IPTG was similar at all points (data not shown). (B) Overexpression of MicL lowers $s^\varepsilon$ activity. Cells with empty vector or pBR*-MicL were induced with 1 mM IPTG when overnight cultures were diluted to OD$_{600}$;0.01. $s^\varepsilon$ activity was measured as in A. Notably, MicL overexpression lowers Lpp protein levels to an extent similar to that observed in ribosome profiling (~10-fold) [cf. Fig. 2C, Supplemental Fig. S10B]. The inset provides the average and standard deviation for increased $s^\varepsilon$ activity for all pBR* and pBR*-MicL points, normalized to pBR* at each time point. (C) Shutoff of $s^\varepsilon$ activity leads to cell death and can be rescued by concomitant expression of MicA or MicL from derivatives of the pEG plasmid. $s^\varepsilon$ activity is shut off by overexpressing the $s^\varepsilon$-negative regulators RseA/B from pTrc-RseAB. Aliquots (2 $\mu$L) of cells growing exponentially in LB with ampicillin (amp) and cm were plated at the indicated dilutions on LB plates $\pm$ 1 mM IPTG, which induces both RseA/B and the sRNA (MicL or MicA).
proteins are chaperoned by the LolA/LolB system. Thus, transient overexpression of Lpp will decrease OM insertion of the BamD/LptE lipoproteins and assembly of their respective machines. This will disrupt LPS and OMP insertion into the membrane, triggering the concomitant accumulation of both LPS and OMPs and \( \sigma^E \) activity.

Together, MicL, MicA, and RybB regulate not only the majority of protein flux targeted to the OM (>85% of the translation of OM proteins) but also a large fraction of total cell protein (~12% of all translation events) [Supplemental Table S3]. As production of OM proteins consumes a large fraction of the cellular resources (~14% of all transcription and translation is devoted to OM proteins] [Supplemental Table S3], \( \sigma^E \) is the regulator of a large section of cellular physiology. Given the central role of these sRNAs in controlling flux of membrane proteins, it is not surprising that their overexpression relieves cell death resulting from insufficient \( \sigma^E \). Although physiological levels of these sRNAs do not fully eliminate Lpp or OMP synthesis, they cause a modest decrease in translation, which nonetheless may have a large effect due to the abundance of these proteins. Even a twofold change in the availability of Lpp mRNA would affect 4% of all translation events and alter the composition of the membrane.

During transition to stationary phase, nutrient limitation severely curtails cell growth, requiring a significantly reduced rate of membrane synthesis. Indeed, we observed a dramatic decrease in the levels of lpp and omp mRNAs during this condition. The necessity of down-regulating new synthesis of Lpp and OMPs may explain why there is a dramatic rise in \( \sigma^E \) activity and the levels of MicL, RybB, and MicA during this transition. As both lpp and omp mRNAs are exceptionally long-lived and well translated, up-regulating these sRNAs simultaneously inhibits new synthesis of these proteins and allows RNases to degrade the mRNAs, thereby facilitating adaptation to stationary phase.

Copper sensitivity is related to lipoprotein biogenesis

We found that cells lacking MicL misregulate Lpp and are sensitive to copper stress. Interestingly, defects in other aspects of lipoprotein homeostasis also lead to increased copper sensitivity. Two additional cut genes, the OM lipoprotein nlpE [cutF] and apolipoprotein N-acetyltransferase Int [cutE] [Gupta et al. 1993], are involved in lipoprotein homeostasis. Lnt is an essential protein that catalyzes lipid attachment to lipoproteins such as Lpp and is the last step in lipoprotein maturation [Narita and Tokuda 2010]. Importantly, \( \Delta lpp \) complements the copper sensitivity of partially defective \( \Delta lnt \) alleles [Gupta et al. 1993] as well as \( \Delta nlpE \) and \( \Delta nlpE \Delta cutC \) [data not shown], suggesting that these copper sensitivity phenotypes reflect Lpp misregulation arising from altered Lpp insertion into the OM or an altered OM environment. Thus, monitoring and controlling Lpp biogenesis is a key component of resistance to copper.

The cutC gene received its name because mutations in the coding sequence conferred sensitivity to copper. Since our investigations establish that this phenotype instead derives from misregulation of MicL and consequent alteration of Lpp biogenesis, the function of CutC should be re-examined. However, it is intriguing that CutC and the YecM protein encoded in the same operon have been hypothesized to be metal-binding proteins [Gupta et al. 1995; Zhang et al. 2003b]. While there is no direct evidence for copper association with bacterial CutC, the conserved human variant of CutC has been shown to bind Cu(I) [Li et al. 2010]. Are the functions of CutC and MicL related and are there advantages of hosting MicL within cutC? Since MicL-S can be processed from the cutC mRNA [Supplemental Fig. S3F], MicL levels could be tied to cutC levels, allowing MicL to be made during exponential phase when \( \sigma^E \) activity is low.

Identification of increasing numbers of 3′ UTR-embedded sRNAs warrants reconsideration of phenotypes attributed to proteins

It is becoming appreciated that sRNAs are not only encoded as independent transcripts in intergenic regions but also originate from within coding regions. sRNAs can be generated by the processing of a larger transcript, as in the case of s-SodF in Streptomyces coelicolor [Kim et al. 2014], or transcribed as a primary transcript like MicL [described here] and DapZ in S. enterica [Chao et al. 2012]. Intriguingly, many of the other candidate 3′ UTR-embedded sRNAs identified in S. enterica [Chao et al. 2012] can be observed in our data set. The fact that the majority of these sRNA transcripts are associated with Hfq strongly implies that they are functional [Chao et al. 2012].
Materials and methods

Strains and plasmids

The bacterial strains and plasmids used in the study are listed in Supplemental Tables S4 and S5, respectively. Gene knock-outs or mutants were constructed in strain NM500 or NM400 using a Red-mediated recombination with DNA fragments generated by PCR using oligonucleotides listed in Supplemental Table S6 [Datzenko and Wanner 2000; Yu et al. 2000; Court et al. 2003]. The mutations linked to markers flanked by FRT sites were moved into new backgrounds by P1 transduction, and, where indicated, antibiotic resistance markers were removed using plasmid pCP20 [Cherepanov and Wackernagel 1995]. For the lpp-lacZ translational fusions (and mutant derivatives), the entire 5′ UTR, beginning with the major lpp transcription start at position 1,755,407 to the indicated position in the coding sequence, was fused to the coding sequence of lacZ behind a pBAD promoter (Mandin and Gottesman 2009). A second lpp promoter was annotated in EcoCyc at position 1,755,320, but only a very weak signal was detected in our deep sequencing analysis [MK Thomason, T Bischler, SK Eisenhart, KU Förster, A Zhang, A Herbig, K Nieselt, CM Sharma, G Storz, in prep.]. In all cases, point mutations were introduced in the fragments used for recombination using overlapping PCR as described previously (Ho et al. 1989).

For plasmid construction, the desired gene fragments were generated by PCR amplification using MG1655 genomic DNA as a template and, after digestion with restriction enzymes, were cloned into the corresponding sites of the indicated vectors. pBR* is a derivative of the pBR322-derived pBRplac vector (here denoted as pBR) [Guillier and Gottesman 2006] in which the ampicillin cassette was replaced by the kanamycin cassette. pBR contains both the ampicillin and the kanamycin cassettes. We found transforming with pBR*-MicL to be more efficient than transforming with pBR-MicL, possibly due to the effects of kanamycin versus ampicillin. All cloning was performed using E. coli TOP10 cells [Invitrogen], and all mutations and plasmid inserts were confirmed by sequencing.

Growth conditions

Unless indicated otherwise, strains were grown aerobically at 37°C in either LB (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter) or EZ rich defined medium (MOPS, Teknova). The copper sensitivity was monitored on LB plates supplemented with 4 mM CuSO₄ (diluted from 1 M stock solution, Sigma) and incubated overnight at 30°C [Tetaz and Luke 1983; Gupta et al. 1985]. Where indicated, IPTG was added at a final concentration of 1 mM or as noted, and antibiotics and chemicals were added when appropriate at the following concentrations: 100 μg mL⁻¹ ampicillin, 30 μg mL⁻¹ kanamycin, 12.5 μg mL⁻¹ tetracycline, 25 μg mL⁻¹ chloramphenicol, or 1.4 mM dibucaine.

Tiling array analysis

 Cultures of E. coli carrying the α5 overexpression plasmid (pPpoE) were grown to OD₆₀₀ = 0.3 at 30°C in LB, and post-induction (0 min) and post-induction (20 min) samples were harvested. After RNA extraction with hot phenol chloroform as described [Massé et al. 2003], each sample was hybridized to a custom Affymetrix E. coli tiling array, and an antibody specific for RNA–DNA complexes detected “ON” tiles as described [Hu et al. 2006]. The tiling array tools provided by Affymetrix, tiling analysis software (TAS) and the integrated genome browser (JGB), were used to analyze the data set.

Deep sequencing and analysis

mRNA-seq and ribosome profiling were performed as previously described, with a few modifications [Ingolia et al. 2009; Li et al. 2012]. Briefly, cells were grown in MOPS to OD = 0.3 and induced with 1 mM IPTG; at the indicated times, 200 mL of cells was harvested. Two replicates were performed for all MicL experiments, with high levels of correlation between experiments. For RNA-seq, the cell pellet was phenol-extracted, and ribosomal RNA was removed with the MICROBExpress kit (Life Technologies). tRNAs were not removed to recover the small RNAs of the cell. For ribosome profiling, ribosome-protected fragments were generated as previously described, yielding 25- to 40-nt footprints [Ingolia et al. 2009; Oh et al. 2011]. rRNA was removed, samples were converted to a sequencing library [Ingolia et al. 2009; Li et al. 2012], and sequencing was performed...
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on an Illumina HiSeq 2000 and aligned to NC_000913.fna (MG1655), allowing for one mismatch.

Analysis was restricted to genes with >128 total counts, a cutoff determined empirically to prevent false positives [Ingolia et al. 2009]. Mean mRNA density and ribosome density were calculated excluding the 5′ and 3′ UTRs and were corrected for total number of reads and the length of each gene and reported in reads per kilobase per million (RPKM) [Ingolia et al. 2009]. Translation efficiency was calculated on a gene-by-gene basis, where translation efficiency was the ratio of ribosome footprints to mRNA fragments for that gene (mean translation/mean expression) [Ingolia et al. 2009]. To calculate each fraction of the total mRNA and translation that each protein represents, the total number of reads per coding region was divided by the total number of reads across all coding regions. All of the deep sequencing data sets are available at Gene Expression Omnibus (GSE58637).

Northern analysis

For Northern analysis, total RNA was extracted by hot acid phenol as described previously [Masse et al. 2003], with minor modifications. Briefly, cells in 1.5 mL of culture (the equivalent of OD$_{600}$-3) were collected, resuspended in 650 µL of buffer A (0.5% SDS, 20 mM NaOAc, 10 mM EDTA), and immediately added to 750 µL of hot acid phenol chloroform (pH 4.5; Ambion). The mixture was vortexed vigorously and incubated for 10 min at 65°C. The sample was then centrifuged at 30,000 rpm for 10 min, and the upper aqueous phase was subjected to another round of hot acid phenol chloroform treatment. The aqueous phase from the second acid phenol extraction was added to 1 mL of 100% ethanol containing 1 m glycogen and precipitated at -20°C incubation at 45/C0. X-ray film at -80°C allowed to air dry for 5 min, and exposed to KODAK Biomax imager (Licor).

For hybridization, membranes were UV cross-linked based on OD 260.

Illumina HiSeq 2000 and aligned to NC_000913.fna (MG1655), allowing for one mismatch.

Promoter activity assays

The MicL promoter-GFP fusion was constructed as described previously [Mutalik et al. 2009], placing the $p_{micL}$ −65 to +20 sequences in front of GFP. Other promoter-GFP fusions are from other laboratories, including a $p_{rpoHp3}$-Lpp antibody (kindly provided by the laboratory of T. Silhavy) followed by incubation with a 1:20,000 dilution of α-GroEL mouse monoclonal [Abcam] followed by incubation with a 1:40,000 dilution of HRP goat anti-mouse IgG [Abcam]. For both Lpp and GroEL, the membranes were developed using SuperSignal West Pico chemiluminescent substrate [Thermo Scientific] and exposed to KODAK Blue-XB film.

For $\beta$-d-Galactosidase assays, we measured $\beta$-galactosidase activity. Four independent experiments were performed for each strain. For Figure 7B, a mean and standard deviation of $p_{micL}$ to $\beta$-d-Galactosidase activity was measured from a $p$-Lpp-Hfq-LacZ reporter as described previously [Ades et al. 1999, Costanzo and Ades 2006]. Briefly, cells were grown to OD$_{600}$-0.1 in LB at 30°C. Four samples were taken at different times, and the $\beta$-galactosidase activities of these samples were plotted against their OD$_{600}$. The slope of this plot represents $\beta$ activity. Four independent experiments were performed for each strain.
Mutarlik et al. (2009). GFP fluorescence was measured using a Varioskan (Thermo) as previously described (Mutarlik et al. 2009). Briefly, promoter strength is a function of the fluorescence and the cell density. GFP fluorescence was measured at four ODs after α induction, and the fluorescence was plotted versus OD. The slope of the linear portion of this plot is reported as the promoter activity of the specific promoter-GFP fusion in that reporter strain.

Acknowledgments

We thank A. Zhang for assistance with the tiling array analysis, S. Gottesman for plasmids and strains, T. Silhavy for sharing Lpp antiserum, and G.-W. Li and D. Burkhardt for helpful discussions. Work in the Gross laboratory was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (ZIA HD001608-23). This work was also supported by the Intramural Research Program of the National Library of Medicine.

References

Ades SE, Connolly LE, Alba BM, Gross CA. 1999. The Escherichia coli α-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an α-factor. Genes Dev 13: 2449–2461.

Barchinger SE, Ades SE. 2013. Regulated proteolysis: control of the Escherichia coli α-dependent cell envelope stress response. Subcell Biochem 66: 129–160.

Beisel CL, Storz G. 2011. The base-pairing RNA Spot 42 participates in a multioutput feedforward loop to help enact catalytic repression in Escherichia coli. Mol Cell 41: 286–297.

Beisel CL, Updegrove TB, Janson BJ, Storz G. 2012. Multiple factors dictate target selection by Hfq-binding small RNAs. EMBO J 31: 1961–1974.

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J. 2008. Small RNA binding to 5’ mRNA coding region inhibits translational initiation. Mol Cell 32: 827–837.

Braun V, Rehn K. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the E. coli cell wall. The specific effect of trypsin on the membrane structure. Eur J Biochem 10: 426–438.

Braun M, Silhavy TJ. 2002. Imp/OstA is required for cell envelope biogenesis in Escherichia coli. Mol Microbiol 45: 1289–1302.

Cezairliyan BO, Sauer RT. 2009. Control of Pseudomonas aeruginosa AlgW protease cleavage of MucA by peptide signals and MucB. Mol Microbiol 72: 368–379.

Chaba R, Grigorova IL, Flynn JM, Baker TA, Gross CA. 2007. Design principles of the proteolytic cascade governing the α5-mediated envelope stress response in Escherichia coli: keys to graded, buffered, and rapid signal transduction. Genes Dev 21: 124–136.

Chaba R, Alba BM, Guo MS, Sohn J, Ahuja N, Sauer RT, Gross CA. 2011. Signal integration by DegS and RseB governs the α5-mediated envelope stress response in Escherichia coli. Proc Natl Acad Sci 108: 106–111.

Chang TW, Lin YM, Wang CF, Liao YD. 2012. Outer membrane lipoprotein Lpp is Gram-negative bacterial cell surface receptor for cationic antimicrobial peptides. J Biol Chem 287: 418–428.

Chao Y, Papenfort K, Reinhardt R, Sharma CM, Vogel J. 2012. An atlas of Hfq-bound transcripts reveals 3’ UTRs as a genomic reservoir of regulatory small RNAs. EMBO J 31: 4005–4019.

Cherepanov PP, Wackernagel W. 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158: 9–14.

Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M. 2010. Micα sRNA links the PhoP regulon to cell envelope stress. Mol Microbiol 76: 467–479.

Costanzo A, Ades SE. 2006. Growth phase-dependent regulation of the extracytoplasmic stress factor, α5, by guanosine 3’,5’-bispyrophosphate (ppGpp). J Bacteriol 188: 4627–4634.

Court DL, Swaminathan S, Yu D, Wilson H, Baker T, Bubunenko M, Sawitzke J, Sharan SK. 2003. Mini-λ: a tractable system for chromosome and BAC engineering. Gene 315: 63–69.

Cowles CE, Li Y, Semmelhack MF, Cristea IM, Silhavy TJ. 2011. The free and bound forms of Lpp occupy distinct subcellular locations in Escherichia coli. Mol Microbiol 79: 1168–1181.

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci 97: 6640–6644.

De La Peñas A, Connolly LE, Gross CA. 1997. α5 is an essential α factor in Escherichia coli. J Bacteriol 179: 6862–6864.

Desnoyers G, Bouchard MP, Massé E. 2013. New insights into small RNA-dependent translational regulation in prokaryotes. Trends Genet 29: 92–98.

Eldar A, Elowitz MB. 2010. Functional roles for noise in genetic circuits. Nature 467: 167–173.

Galdiero S, Falanga A, Cantisani M, Tarollo R, Della Pepa ME, D’Oriano V, Galdiero M. 2012. Microbe-host interactions: structure and role of Gram-negative bacterial porins. Curr Protein Pept Sci 13: 843–854.

Gogol EB, Rhodius VA, Papenfort K, Vogel J, Gross CA. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. Proc Natl Acad Sci 108: 12875–12880.

Guillier M, Gottesman S. 2006. Remodelling of the Escherichia coli outer membrane by two small regulatory RNAs. Mol Microbiol 59: 231–237.

Guisbert E, Rhodius VA, Ahuja N, Witkin E, Gross CA. 2007. Hfq modulates the α5-mediated envelope stress response and the α5-mediated cytoplasmic stress response in Escherichia coli. J Bacteriol 189: 1963–1973.

Gupta SD, Gan K, Schmid MB, Wu HC. 1993. Characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in apolipoprotein N-acyltransferase. J Biol Chem 268: 16551–16556.

Gupta SD, Lee BT, Camakaris J, Wu HC. 1995. Identification of cutC and cutF [nlpE] genes involved in copper tolerance in Escherichia coli. J Bacteriol 177: 4207–4215.

Hayden JD, and Ades SE [2008]. The extracytoplasmic stress factor, α5, is required to maintain cell envelope integrity in Escherichia coli. PLoS ONE 3: e1573.

Hirot a Y, Suzuki H, Nishimura Y, Yasuda S. 1977. On the process of cellular division in Escherichia coli lacking a murein-lipoprotein. J Bacteriol 129: 420–427.

Hu Z, Zhang A, Storz G, Gottesman S, Leppa SH. 2006. An antibody-based microarray assay for small RNA detection. Nucleic Acids Res 34: e52.

This work was also supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of General Medical Science (GM036278), and work in the Gross laboratory was supported by National Institutes of Health Grants GM08853 and GM062902. We thank A. Zhang for assistance with the tiling array analysis, S. Gottesman for plasmids and strains, T. Silhavy for sharing Lpp antiserum, and G.-W. Li and D. Burkhardt for helpful discussions. Work in the Gross laboratory was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (ZIA HD001608-23). This work was also supported by the Intramural Research Program of the National Library of Medicine.
Ingle CA, Kushner SR. 1996. Development of an in vitro mRNA decay system for Escherichia coli: poly(A) polymerase I is necessary to trigger degradation. Proc Natl Acad Sci 93: 12926–12931.

Ingolia NT, Ghaemmaghami S, Newman JRS, Weissman JS. 2009. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 324: 218–223.

Inouye M, Shaw J, Shen C. 1972. The assembly of a structural lipoprotein in the envelope of Escherichia coli. J Biol Chem 247: 8154–8159.

Johansen J, Rasmussen AA, Overgaard M, Valentin-Hansen P. 2006. Conserved small non-coding RNAs that belong to the σ5 regulon: role in down-regulation of outer membrane proteins. J Mol Biol 364: 1–8.

Johansen J, Eriksen M, Kallipolitis B, Valentin-Hansen P. 2008. Down-regulation of outer membrane proteins by noncoding RNAs: unraveling the cAMP-CRP- and σ5-dependent CyaR-ompX regulatory case. J Mol Biol 383: 1–9.

Kim HM, Shin JH, Cho YB, Roe JH. 2014. Inverse regulation of Fe- and Ni-containing SOD genes by a Fur family regulator Nur through small RNA processed from 3′UTR of the sodF mRNA. Nucleic Acids Res 42: 2003–2014.

Levine E, Zhang Z, Kuhlman T, Hwa T. 2007. Quantitative characteristics of gene regulation by small RNA. PLoS Biol 5: e229.

Li Y, Du J, Zhang P, Ding J. 2010. Crystal structure of human copper homeostasis protein CucT reveals a potential copper-binding site. J Struct Biol 169: 399–405.

Li G-W, Oh E, Weissman JS. 2012. The anti-Shine-Dalgarno sequence drives translational pausing and codon choice in Salmonella. mBio 3: 587–595.

Li G-W, Burkhardt DH, Gross CA, Weissman JS. 2014. Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. Cell 157: 624–635.

Lima S, Guo MS, Chaba R, Gross CA, Sauer RT. 2013. Dual molecular signals mediate the bacterial response to outer-membrane stress. Science 340: 837–841.

Mandin P, Gottesman S. 2009. A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. Mol Microbiol 72: 551–565.

Masse E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli. Genes Dev 17: 2374–2383.

Mathew S, Carlini J, Li Z, Waltzer P, Gottesman S. 2007. Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res 35: e63.

Mecas J, Rouviere PE, Erickson JW, Donohue TJ, Gross CA. 1993. The activity of σ5, an Escherichia coli heat-inducible σ-factor, is modulated by expression of outer membrane proteins. Genes Dev 7: 2618–2628.

Mizuno T, Chou MY, Inouye M. 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (microRNA). Proc Natl Acad Sci 81: 1966–1970.

Mutalik VK, Nonaka G, Ades SE, Rhodius VA, Gross CA. 2009. Promoter strength properties of the complete σE regulon of Escherichia coli and Salmonella enterica. J Bacteriol 191: 2797–2797.

Nair A, Tokuda H. 2010. Biogenesis and membrane targeting of lipoproteins. EcoSal Plus doi: 10.1128/ecosalplus.A4.3.7.

Nichols RJ, Sen S, Choo YJ, Beltrao P, Zietek M, Chaba R, Lee S, Kazmierczak KM, Lee KJ, Wong A, et al. 2011. Phenotypic landscape of a bacterial cell. Cell 144: 143–156.

Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67: 593–656.

Nilsson G, Belasco JC, Cohen SN, von Gabain A. 1984. Growth-rate dependent regulation of mRNA stability in Escherichia coli. Nature 312: 75–77.

Oh E, Becker AH, Sandicki A, Huber D, Chaba R, Gloge F, Nichols RJ, Typas A, Gross CA, Kramer G, et al. 2011. Selective ribosome profiling reveals the cotranslational chaperone action of trigger factor in vivo. Cell 147: 1295–1308.

Opdyke JA, Fozo EM, Hemm MR, Storz G. 2011. RNAse III participates in GądY-dependent cleavage of the gädX-gädW mRNA. J Mol Biol 406: 29–43.

Papenfort K, Pleifer V, Mika F, Lucchini S, Hinton JC, Vogel J. 2006. σ5-Dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. Mol Microbiol 62: 1674–1688.

Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J. 2010. Evidence for an autonomous 5′ target recognition domain in an Hfq-associated small RNA. Proc Natl Acad Sci 107: 20435–20440.

Park SH, Bao Z, Butcher BG, D’Amico K, Xu Y, Stodghill P, Schneider DJ, Cartinhour S, Filiatrault ML. 2014. Analysis of the small RNA spf in the plant pathogen Pseudomonas syringae pv. tomato strain DC3000. Microbiology 160: 941–953.

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P. 2005. Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. Mol Microbiol 58: 1421–1429.

Rhodius VA, Suh WC, Nonaka G, West J, Gross CA. 2006. Conserved and variable functions of the σ5 stress response in related genomes. PLoS Biol 4: e23.

Rhodius VA, Mutalik VK, Gross CA. 2012. Predicting the strength of UP-elements and full-length E. coli σ5 promoters. Nucleic Acids Res 40: 2907–2924.

Ricci DP, Silhavy TJ. 2012. The Bam machine: a molecular cooper. Biochim Biophys Acta 1818: 1067–1084.

Richards GR, Vanderpool CK. 2011. Molecular call and response: the physiology of bacterial small RNAs. Biochim Biophys Acta 1809: 525–531.

Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb Perspect Biol 2: a000414.

Skovierova H, Rowley G, Rezuchova B, Homorova D, Lewis C, Roberts M, Kormaneck J. 2006. Identification of the σ5 regulon of Salmonella enterica serovar Typhimurium. Microbiology 152: 1347–1359.

Storz G, Vogel J, Wassarman KM. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Mol Cell 43: 880–891.

Suzuki H, Nishimura Y, Yasuda S, Nishimura A, Yamada M, Hirota Y. 1978. MurE-lipoprotein of Escherichia coli: a protein involved in the stabilization of bacterial cell envelope. Mol Gen Genet 167: 1–9.

Tetaz TJ, Luke RK. 1983. Plasmid-controlled resistance to copper in Escherichia coli. J Bacteriol 154: 1263–1268.

Thomason MK, Fontaine F, De Lay N, Storz G. 2012. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. Mol Microbiol 84: 17–35.

Thompson KM, Rhodius VA, Gottesman S. 2007. σ5 regulates and is regulated by a small RNA in Escherichia coli. J Bacteriol 189: 4243–4256.

Udekwu KI, Wagner EGH. 2007. σ5 Controls biogenesis of the antisense RNA MicA. Nucleic Acids Res 35: 1279–1288.

Udekwu KI, Darfeuille F, Vogel J, Reimega ˚rd J, Holmqvist E, Wagner EGH. 2005. Hfq-dependent regulation of OmpA.
synthesis is mediated by an antisense RNA. *Genes Dev* 19: 2355–2366.

Vogel J, Luisi BF. 2011. Hfq and its constellation of RNA. *Nat Rev Microbiol* 9: 578–589.

Walsh NP, Alba BM, Bose B, Gross CA, Sauer RT. 2003. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* 113: 61–71.

Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D. 2005. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* 121: 235–245.

Yakushi T, Tajima T, Matsuyama S, Tokuda H. 1997. Lethality of the covalent linkage between mislocalized major outer membrane lipoprotein and the peptidoglycan of *Escherichia coli*. *J Bacteriol* 179: 2857–2862.

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci* 97: 5978–5983.

Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S. 2003a. Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* 50: 1111–1124.

Zhang RG, Duke N, Laskowski R, Evdokimova E, Skarina T, Edwards A, Joachimiak A, Savchenko A. 2003b. Conserved protein YecM from *Escherichia coli* shows structural homology to metal-binding isomerases and oxygenases. *Proteins* 51: 311–314.

Zhang G, Meredith TC, Kahne D. 2013. On the essentiality of lipopolysaccharide to Gram-negative bacteria. *Curr Opin Microbiol* 16: 779–785.