REVIEW

# Modulating the outer membrane with small RNAs

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**MicF, one of the first chromosomally encoded regulatory small RNAs (sRNAs) to be discovered, was found to modulate the expression of OmpF, an abundant outer membrane protein. Several recent papers have now shown that this is not an isolated case. At least five other sRNAs also regulate the synthesis of outer membrane porins, and additional sRNAs modulate the expression of other outer membrane proteins. Here we review what is known about these sRNAs and discuss the implications of this regulation.**

## *Escherichia coli* small RNAs (sRNAs)

Small untranslated regulatory RNAs, usually referred to as noncoding RNAs (ncRNAs) in eukaryotes and sRNAs in bacteria, have been described in all kingdoms of life. Regulatory sRNAs in bacteria were first detected from extrachromosomal genetic elements such as plasmids, transposons, and bacteriophages (for review, see Wagner et al. 2002). Subsequently, several studies showed that chromosomally encoded sRNAs, most initially found fortuitously, were induced by and contributed to responses to stress conditions such as low temperature and oxidative stress (for review, see Gottesman 2004). The recognition that sRNAs had important regulatory functions led several groups to carry out genome-wide searches for these regulatory molecules. To date, a variety of approaches (for review, see Vogel and Sharma 2005) have led to the identification of ~80 sRNAs in *E. coli*. The precise role of most of the sRNAs is still unknown, though it appears that many are only expressed under specific conditions.

Thus far, two main modes of action have been described for regulatory sRNAs in *E. coli*. Some modify the activity of a protein, as has been shown for the CsrB and CsrC RNAs (Liu et al. 1997; Weilbacher et al. 2003). These two sRNAs each possess multiple binding sites for the global translational regulatory protein CsrA; upon expression they titrate CsrA away from its mRNA target sites. Another mechanism for sRNA action is direct base-pairing with an mRNA target, either with extensive complementarity to an mRNA encoded in *cis* or with partial complementarity to an mRNA encoded in *trans*. Base-pairing of the sRNA and its target has been found to positively or negatively affect stability and/or the ability of the mRNA to be translated. For instance, both the DsrA and RprA sRNAs activate *rpoS* translation because their base-pairing with the *rpoS* mRNA leader prevents the formation of an inhibitory structure that sequesters the ribosome-binding site (Majdalani et al. 1998, 2002). In contrast, RyhB RNA pairing with the *sodB* mRNA blocks translation and leads to degradation of both RNAs (Massé et al. 2003). This degradation, which requires the RNase E endonuclease, may be secondary to the translation block, since reduced SodB protein levels upon RyhB expression are still observed in the absence of degradation (Morita et al. 2006).

All *E. coli* sRNAs that were shown to act by pairing with *trans*-encoded targets bind the RNA chaperone protein Hfq and, where it was examined, were found to require this protein for their activity. In addition, all Hfq-binding sRNAs studied thus far have been found to act by pairing (for review, see Storz and Gottesman 2006). Hfq was first described as a host-factor required for replication of bacteriophage Qβ, but the pleiotropic effects displayed by an *hfq*-null mutant suggested important cellular roles for this highly abundant protein (Tsui et al. 1994). The hexameric Hfq ring has been shown to bind AU-rich sequences in the sRNAs as well as some target mRNAs, and to stabilize some of the RNAs, probably by masking RNase E cleavage sites. In addition, Hfq has been found to facilitate the interaction between sRNAs and mRNAs, though the mechanism by which this occurs remains to be fully elucidated (for review, see Valentin-Hansen et al. 2004). Since at least one third of the known *E. coli* sRNAs can bind Hfq, base-pairing with target mRNAs appears to be a major mechanism for sRNA action.

sRNAs that act by pairing with *trans*-encoded mRNAs may require the Hfq chaperone because they only have partial complementarity with their targets. An advantage of partial complementarity is that *trans*-encoded sRNAs can regulate the expression of several unlinked

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genes, as was shown to be the case for RyhB, which negatively regulates the expression of multiple operons encoding iron-binding proteins (Massé and Gottesman 2002; Massé et al. 2005). Conversely, the expression of a given gene can be modulated by distinct *trans*-encoded sRNAs. For example, *rpoS* expression is regulated by at least three sRNAs (for review, see Repoila et al. 2003).

It is becoming increasingly clear that base-pairing sRNAs are widespread regulators of genetic expression in bacteria. This mode of regulation has several major advantages for the cell. Synthesis of sRNA regulators is rapid and requires less energy input than the synthesis of protein regulators. In addition, a given sRNA can act at multiple targets, in response to input signals distinct from those regulating transcription of the target; multiple sRNAs can also act on a single target and provide the integration of different input signals. Degradation of the mRNA clearly makes the process irreversible; it is less clear whether translational repression or activation, in the absence of degradation, is a reversible process.

Investigations of the regulation and targets of sRNAs have recently uncovered a major role for these molecules in the modulation of the bacterial cell surface, in particular, outer membrane proteins of Gram-negative bacteria. Here we introduce the critical characteristics of the outer membrane, followed by a consideration of the sRNAs that regulate outer membrane proteins.

## The *E. coli* outer membrane

All Gram-negative bacterial cells are surrounded by a cell envelope, which is comprised of an inner membrane, a periplasmic space, and an outer membrane (for review, see Ruiz et al. 2006). The inner membrane is a lipid bilayer composed mainly of phospholipids and proteins. Proteins found in the inner membrane are primarily involved in metabolic processes such as oxidative phosphorylation, protein translocation, and small-molecule transport and sensing. The interstitial periplasmic space, an oxidizing environment lacking ATP that can comprise as much as 10% of the cell volume, contains a thin peptidoglycan layer and soluble proteins. Functions of periplasmic proteins include the transport of small molecules and the breakdown of polymers. The outer membrane is a very asymmetric lipid bilayer with the inner leaflet composed of phospholipids and the outer leaflet composed of lipopolysaccharides (LPS). Proteins found associated with the outer membrane are either lipoproteins, which are attached to the periplasmic side of the membrane by lipid modifications, or integral outer membrane proteins, which span the membrane with amphipathic antiparallel β-strands that adopt a barrel-like conformation.

The outer membrane serves a number of important roles for the cell. First and foremost it represents a barrier to the entry and exit of both beneficial and harmful molecules. Many of the proteins in the outer membrane function to control the movement of molecules; they facilitate the uptake of nutrients and excretion of toxic molecules while also preventing the entry of deleterious molecules (for review, see Nikaido 2003). Among the most abundant outer membrane proteins are the OmpC and OmpF porins, both trimeric β-barrel proteins that span the membrane. OmpA, another abundant β-barrel protein, acts as a porin (Sugawara and Nikaido 1992), even though its role in the total permeability of the cell is likely negligible compared to OmpC and OmpF. OmpA has also been implicated in stabilizing the cell envelope structure (Sonntag et al. 1978). While OmpF and OmpC, and probably OmpA, serve as general entry portals for small molecules, other related β-barrel outer membrane proteins act as specific channels, often for larger nutrients such as vitamin B12 and iron–siderophore complexes (for review, see Nikaido 2003). Transport through these channels, sometimes referred to as gated channels, is coupled to TonB, which is part of a complex of proteins found in the inner membrane that couple the gated channels to the protonmotive force, the energy source for transport (for review, see Postle and Kadner 2003). Yet other outer membrane proteins serve as anchors for cell surface organelles such as pili.

With their accessibility on the outside of the bacterial cell, outer membrane proteins also represent attachment sites for bacteriophages and colicins. In addition, outer membrane proteins and structures such as flagella and LPS are the features recognized by the immune system of eukaryotic cells and in some cases are required for host cell interactions.

Given the importance of the outer membrane, it is not surprising that the expression of outer membrane proteins has been found to be under complex transcriptional regulation. Two key regulators are the inner membrane sensor kinase EnvZ and the cognate response regulator OmpR (for review, see Pratt et al. 1996). EnvZ autophosphorylates in response to stimuli, such as high osmolarity, that appear to perturb the outer membrane environment. EnvZ then transfers its phosphate group to OmpR. Depending on its phosphorylation state, OmpR has different binding affinities for different promoters. Thus, at low osmolarity OmpR activates *ompF* transcription, whereas at high osmolarity OmpR represses *ompF* transcription and activates *ompC* transcription. This regulation is consistent with what might be expected for the porins. Under high osmolarity conditions, such as in environments inside a host where nutrient levels are high, the small pore porin OmpC will predominate, thus limiting the entry of toxic compounds such as bile salts. Conversely, under low osmolarity conditions, such as in an ex vivo environment scarce in nutrients, OmpF will be the major porin, its larger pore allowing more efficient entry of nutrients (for review, see Nikaido 2003).

The specific gated-channel proteins are generally only expressed under conditions when they might be needed. As an example, most gated channels for iron–siderophore complexes are regulated by iron availability as part of the Fur regulon (for review, see Hantke and Braun 1997). When iron is abundant, the corresponding genes are repressed; when iron is limiting, channels are expressed.

Another layer of regulation occurs in response to de-

fects in the transport and assembly of the outer membrane proteins. The outer membrane proteins are synthesized in the cytoplasm with N-terminal signal sequences. These signal sequences allow transport across the inner membrane via an inner membrane translocon complex composed of SecY, SecE, and SecG (for review, see Bernstein 2000). For lipoproteins, lipid attachment and signal sequence cleavage after translocation produces the mature, membrane-bound lipoproteins. Specific chaperones are necessary for the periplasmic transport and outer membrane attachment of lipoproteins, while other chaperones, together with a partially characterized complex of proteins, transfer and assemble the β-barrel proteins in the outer membrane (for review, see Ruiz et al. 2006). Accumulation of unfolded outer membrane proteins in the periplasm, presumably reflecting problems in protein transport to the outer membrane, is sensed by a regulatory cascade comprised of the alternative σ factor $\sigma^E$, the anti-σ factor RseA, and a number of periplasmic proteases (for review, see Ruiz and Silhavy 2005). In the absence of cell envelope stress, the inner membrane-bound RseA protein sequesters $\sigma^E$. Conditions that lead to the accumulation of misfolded proteins in the periplasm activate proteases that degrade RseA, leading to the release of $\sigma^E$ and the activation of $\sigma^E$-dependent genes, many of which encode protein chaperones, including those necessary for the export and assembly of the outer membrane proteins. Other $\sigma^E$-dependent genes encode periplasmic proteases, which are necessary to reduce the accumulation of misfolded proteins. Mutations that prevent $\sigma^E$ induction have profound effects on cell growth, but can be relieved by decreasing the synthesis of outer membrane proteins (Douchin et al. 2006). This complex regulatory network suggests that the accumulation of proteins beyond what can be properly assembled in the outer membrane presents a significant problem to the cell.

## Regulating OmpF, OmpC, and OmpA with sRNAs

In 1984, MicF, the first base-pairing RNA encoded by the *E. coli* genome to be discovered, was shown to regulate the expression of the major outer membrane porin, OmpF (Mizuno et al. 1984). Twenty years later, with the renewed interest in sRNA regulators, five additional sRNAs that modulate expression of the abundant outer membrane proteins OmpF, OmpC, and OmpA have been found. Here we discuss the features of these sRNAs; a summary of their characteristics and known targets is given in Table 1, and examples of the pairing interactions are shown in Figure 1.

### *MicF RNA*

The 93-nucleotide (nt) MicF (mRNA-interfering complementary RNA for *ompF* gene) was identified during the characterization of the *ompC* promoter; a DNA fragment carrying the region located upstream of the *ompC* promoter was shown to inhibit OmpF production and to decrease the amount of *ompF* mRNA when cloned on a multicopy plasmid (Mizuno et al. 1984). Closer analysis of this fragment revealed the presence of a transcription unit that was encoded divergent from the *ompC* gene and did not contain an ORF. The 5′ end of the sequence was partially complementary to nucleotides encompassing the ribosome-binding site and AUG start codon of the *ompF* mRNA (Fig. 1A; Andersen et al. 1987), suggesting that MicF might act by pairing with this mRNA. Consistent with this model, MicF was shown to interact in vitro with a fragment of *ompF* mRNA carrying the predicted region of base-pairing (Andersen and Delihas 1990). In addition, in vitro enzymatic and chemical probing of secondary structures of both MicF and an *ompF* mRNA fragment, alone or in complex, supported the existence of an extensive interaction encompassing the predicted region of base-pairing (Schmidt et al. 1995). Later studies showed that Hfq binds to MicF (Zhang et al. 2003), which is consistent with MicF acting by base-pairing.

The transcription of the MicF RNA has been shown to be regulated by multiple signals. Expression is induced at high osmolarity by the OmpR regulator (Ramani et al. 1994); under these conditions, OmpR also negatively regulates *ompF* transcription and stimulates synthesis of OmpC. The SoxS and MarA regulators positively regulate MicF, in response to oxidative stress (Chou et al. 1993) and exposure to certain drugs (Cohen et al. 1988), respectively. Presumably, the induction of MicF by SoxS and MarA and the resulting decrease in OmpF helps limit the entry of toxic or redox-active molecules. MicF RNA levels are also increased by a shift to higher temperatures (Andersen et al. 1989), but neither the regulator nor the physiological consequences of this induction are known. Although many *trans*-encoded sRNAs that bind Hfq regulate multiple mRNAs, *ompF* is the only characterized target of MicF. Future work to identify any additional target mRNAs may help further define the biological significance of MicF action.

### *MicC RNA*

The 109-nucleotide (nt) MicC RNA (previously denoted IS063) was first identified in a computational screen as a sRNA encoded in the *ompN-ydbK* intergenic region, divergent to the *ompN* gene (Chen et al. 2002), which encodes an unexpressed porin homologous to OmpC and OmpF. The *ompN-micC* genetic organization is reminiscent of the *ompC-micF* organization described above, although *ompN* is missing in some pathogenic *E. coli* strains that still contain *micC*. A BLAST search for sequences complementary to MicC in *E. coli* suggested that the sRNA could base-pair with the *ompC* mRNA just upstream of the ribosome-binding site, thus making *ompC* a good candidate for MicC regulation (Fig. 1B). Indeed, Chen et al. (2004) showed that MicC negatively regulates *ompC* expression at the post-transcriptional level by base-pairing with *ompC* mRNA and preventing the formation of an active translation initiation complex. MicC is also bound by Hfq, and Hfq is required for MicC regulation of OmpC levels.

**Table 1.** *sRNA regulators of outer membrane proteins*

| Name | Alternate names | Flanking genes | Ig length | Strand | sRNA size | Regulators | Expression | Hfq[a] | Targets | Conservation[b] | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MicF | | *ompC/yojN* | 738 | < > > | 93 | MarA/Sox/Rob | High osmolarity, superoxide, heat | Yes | *ompF* | Ec, Sa, Kp, Sm, Yp | Mizuno et al. 1984; Delihas and Forst 2001 |
| MicC | IS063 | *ompN/ydbK* | 369 | < > < | 109 | Unknown | Opposite of MicF? | Yes | *ompC* | Ec, Sa, Sh, Kp | Chen et al. 2002, 2004 |
| MicA | SraD | *luxS (ygaG)/gshA* | 153 | < > < | ~75 | $\sigma^E$ | Stationary | Yes | *ompA* | Ec, Sh, Yp, Ew, Sm, Kp | Rasmussen et al. 2005; Udekwu et al. 2005 |
| RybB | | *ybjK/ybjL* | 180 | > < < | 80 | $\sigma^E$ | Stationary | Yes | *ompC, ompW* | Ec, Ew, Kp, Sa, Sh, Sm, Yp | Vogel et al. 2003; J. Johansen, A.A. Rasmussen, M. Overgaard, and P. Valentin-Hansen, in prep.; K. Thompson and S. Gottesman, in prep. |
| RseX | | *yedR/ompS (yedS)* | 518 | < > > | 91 | Unknown | Unknown | Yes | *ompC, ompA* | Ec, Sh, Kp? | Douchin et al. 2006 |
| IpeX | | *ybcQ/nmpC* (phage *qsr'* genome) | 189 | > < < | Unknown | Unknown | Unknown, possibly cotranscribed with *nmpC* | ? | *ompC, ompF* | PA-2 lysogens? | Castillo-Keller et al. 2006 |
| OmrA | RygA, SraE | *aas/(omrB)-galR* | 584 | < < < > | 88 | OmpR | High osmolarity | Yes | *ompT, cirA, fecA, fepA* | Ec, Sa, Sh, Kp | Guillier and Gottesman 2006 |
| OmrB | RygB | *aas-(omrA)/galR* | 584 | < < < > | 82 | OmpR | High osmolarity | Yes | *ompT, cirA, fecA* | Ec, Sa, Sh, Kp,Yp, Ct, Ew | Guillier and Gottesman 2006 |

[a]Immunoprecipitation and/or binding to Hfq demonstrated.
[b](Ct) Citrobacter; (Ec) *E. coli*; (Ew) *Erwinia*; (Kp) *Klebsiella pneumoniae*; (Sa) *Salmonella* species; (Sh) Shigella; (Sm) *Serratia marcescens*; (Yp) *Yersinia pestis*.

Northern analysis revealed that, in general, MicC shows expression that is opposite to MicF (Chen et al. 2004). For example, the levels of MicC are elevated at low temperature and decreased at high temperature. OmpR appears to repress *micC*, either directly or indirectly (Chen et al. 2004); other regulators that impact expression of the sRNA have not been identified. The physiological role of MicC is not known. Is there a specific need to reduce OmpC under some growth conditions, or a requirement for post-transcriptional as well as transcriptional regulation during transitions in growth conditions? Again, the identification of any additional MicC targets as well as further information about MicC expression could help address these questions.

### *MicA RNA*

The mRNA encoding the OmpA porin has long been known to be unusually stable. This stability was studied extensively and shown to be both growth-rate and growth-phase dependent (Nilsson et al. 1984). The first indication that a sRNA could be involved in the growth-phase dependent control of *ompA* transcript stability came from Vytvytska et al. (2000), who reported that Hfq binds *ompA* mRNA and decreases its stability. This decreased stability was originally interpreted as being the result of direct competition between Hfq and 30S ribosomal subunits for *ompA* mRNA binding. However, two recent studies show that this control is actually mediated by the ~75-nt MicA RNA (also denoted SraD) (Rasmussen et al. 2005; Udekwu et al. 2005). Given these findings, it would seem that the stable message is the default situation; when the MicA RNA is expressed, both the stability and translation of the *ompA* message decrease.

MicA was initially found in a computational screen for sRNAs (Argaman et al. 2001). Northern analysis showed that the sRNA is abundant in stationary phase in rich

medium. The possibility that MicA might be regulating OmpA expression was suggested by complementarity between MicA and the 5′ sequence of the *ompA* mRNA (Fig. 1C), as well as by the observation that MicA overexpression leads to reduced OmpA protein levels (Rasmussen et al. 2005; Udekwu et al. 2005). MicA base-pairing overlapping the *ompA* ribosome-binding site was confirmed by in vitro mobility assays and mutational studies and was shown to prevent ribosome binding. This control requires Hfq, and Hfq facilitates the MicA–*ompA* interaction, at least in part, by binding both the *ompA* mRNA and MicA. Consistent with its expression pattern, MicA accounts for the destabilization of the *ompA* transcript in stationary phase in vivo; under these conditions OmpA levels are significantly elevated in a strain deleted for MicA.

Understanding the function of MicA requires knowing when the cell would want to decrease OmpA. OmpA is an extremely abundant and well-conserved outer membrane protein. It has been suggested to have multiple functions, including roles as a barrier and determinant of cell structure as well as a mammalian cell attachment site. OmpA may play both positive and negative roles during infection. *E. coli* strains lacking OmpA have been found to be less virulent in embryonic chicken and neonatal rat models (Weiser and Gotschlich 1991). In addition, OmpA is specifically bound by the major serum amyloid A protein, a mammalian immune response protein (Hari-Dass et al. 2005), and is required for bacterial killing by neutrophil elastase after infection (Belaaouaj et al. 2000). Thus, varied OmpA expression at different stages of in vivo growth may be critical for virulence; for example, reducing its expression at the appropriate time may limit the host immune response.

The regulation of MicA may provide a strong additional hint for why this sRNA may be important. MicA expression was reported to be highest in stationary phase (Argaman et al. 2001). The sequence of the MicA promoter matches the consensus for $\sigma^E$-dependent promoters, and recent studies have shown that indeed MicA induction in stationary phase is dependent on $\sigma^E$ (J. Johansen, A.A. Rasmussen, M. Overgaard, and P. Valentin-Hansen, in prep.; K. Udekwu and G. Wagner, in prep.). Since $\sigma^E$ activity will increase when unfolded outer membrane proteins accumulate, MicA may be part of a network to ensure that such unfolded proteins, including OmpA, are not made if they cannot be properly assembled into the outer membrane.

It is worth noting that OmpA translation has also been observed to change when cells are growing slowly in exponential phase (growth-rate control) (Nilsson et al. 1984). This regulation is not dependent on MicA, but is dependent on Hfq (Vytvytska et al. 1998), suggesting the possibility that yet another sRNA regulates OmpA expression under these conditions. Such a possible split in responsibilities suggests that there may be different reasons for limiting OmpA expression under different growth conditions and/or that under each of these specific conditions corepression of different sets of targets, mediated by different sRNAs, is important.

### *RybB RNA*

Yet another sRNA found to be synthesized in a $\sigma^E$-dependent fashion is the 80-nt RybB RNA, which was initially identified in a computational screen for conserved intergenic sequences (Wassarman et al. 2001; Vogel et al. 2003; J. Johansen, A.A. Rasmussen, M. Overgaard, and P. Valentin-Hansen, in prep.; K. Papenfort, F. Miko, V. Pfeiffer, and J. Vogel, in prep.; K. Thompson and S. Gottesman, in prep.). Since RybB was known to bind Hfq, the sRNA was assumed to act by base-pairing, and possible targets of RybB were identified by carrying out whole genome expression analysis to examine the effect of transiently overexpressing RybB. The levels of the mRNAs encoding OmpC as well as OmpW, another outer membrane protein with homology with OmpC, were decreased in these experiments (J. Johansen, A.A. Rasmussen, M. Overgaard, and P. Valentin-Hansen, in prep.). Northern analysis confirmed that the *ompC* and *ompW* mRNA levels are indeed modulated by RybB and base-pairing between the sRNA and these mRNAs can be predicted (Fig. 1B). Interestingly, RybB also down-regulates $\sigma^E$ activity, although it is not yet clear whether this is a direct effect or an indirect effect (for instance, via down-regulation of the outer membrane proteins) (K. Thompson and S. Gottesman, in prep.).

### *RseX RNA*

The connection between the $\sigma^E$ regulon and sRNA down-regulation of outer membrane proteins is further reinforced by the recent identification of RseX (RNA suppressor of extracytoplasmic stress protease) as another sRNA regulator of OmpA and OmpC expression. RseX was uncovered in a screen for multicopy suppressors of the growth defect associated with the depletion of one of the periplasmic proteases that degrade the RseA anti-σ factor (Douchin et al. 2006). Since RseA keeps $\sigma^E$ activity low, the levels of the $\sigma^E$-regulated periplasmic chaperones required for proper outer membrane protein transport are constitutively low in this background. Several multicopy clones isolated in this screen encoded a 91-nt RNA denoted RseX. The *rseX* gene is on the same strand and upstream of *yedS*, which encodes a putative homolog of OmpS1, an outer membrane protein in *Salmonella typhi*. Hfq binds the sRNA in vitro and is required for the suppression phenotype. The *ompA* and *ompC* transcripts were identified as targets of RseX because they were captured when RNA extracted from *E. coli* was incubated with synthetic RseX (Douchin et al. 2006). RseX is predicted to be capable of fairly extensive base-pairing across the ribosome-binding sites of both *ompA* and *ompC* (Fig. 1B,C), and RseX was shown to interact with *ompA* in mobility shift assays. The down-regulation of OmpA and OmpC expression is sufficient to explain the suppressor phenotype of the RseX clone, since the periplasmic protease deficient strain is also viable when the *ompA* and *ompC* genes are deleted (Douchin et al. 2006). Thus far, the RseX RNA has only been detected in cells with the multicopy clone; conditions

that lead to RseX expression from the chromosome have not been reported.

### *IpeX RNA*

A sixth putative sRNA that has been reported to control porin production, encoded by the gene denoted *ipeX* (inhibitor of porin expression), was also identified as a multicopy suppressor. The plamid carrying *ipeX* suppressed the lethality associated with production of a mutant form of OmpC, $OmpC_{2Cys}$, expressed in the absence of a $\sigma^E$-regulated periplasmic protease, DegP (Castillo-Keller et al. 2006). The IpeX RNA was shown to restore the viability of this mutant by inhibiting the production of $OmpC_{2Cys}$. However, this effect was not specific to $OmpC_{2Cys}$, since IpeX also inhibited the production of wild-type OmpC and to a lesser extent OmpF. IpeX is encoded on the same strand as and downstream of *nmpC* in the *ybcQ-nmpC* intergenic region, and is part of the genome of a cryptic phage *qsr'* present in several *E. coli* strains. Interestingly, *nmpC* encodes a porin, although the *nmpC* gene is interrupted by an IS5 element and thus is not expressed in many laboratory *E. coli* strains. However, the *ipeX* sequence is also found in the genome of PA-2 phage, downstream of the *lc* gene, encoding a phage porin. In that case, *ipeX* is transcribed as a part of the *lc* gene and may be processed from this longer transcript. An intriguing possibility is that production of the phage porin results in the decrease of the host porin OmpC levels through the coupled transcription of *lc* and *ipeX*. Indeed, it was reported more than 30 years ago that *E. coli* strains lysogenized with PA-2 have reduced OmpC levels (Schnaitman et al. 1975).

Though the IpeX RNA acts at a post-transcriptional level to down-regulate both OmpC and OmpF, it may be acting differently than MicC and MicF. It was reported that IpeX-mediated destabilization of the *ompC* mRNA is independent of Hfq and that there is only limited potential for base-pairing between *ipeX* and the *ompC* and *ompF* mRNAs (Castillo-Keller et al. 2006). However, an extended region of complementarity between the IpeX RNA and *ompC* mRNA is predicted by a program for detecting sRNA–mRNA interactions (Fig. 1B; Tjaden et al. 2006) Additional experiments will be necessary to address whether IpeX also acts by pairing.

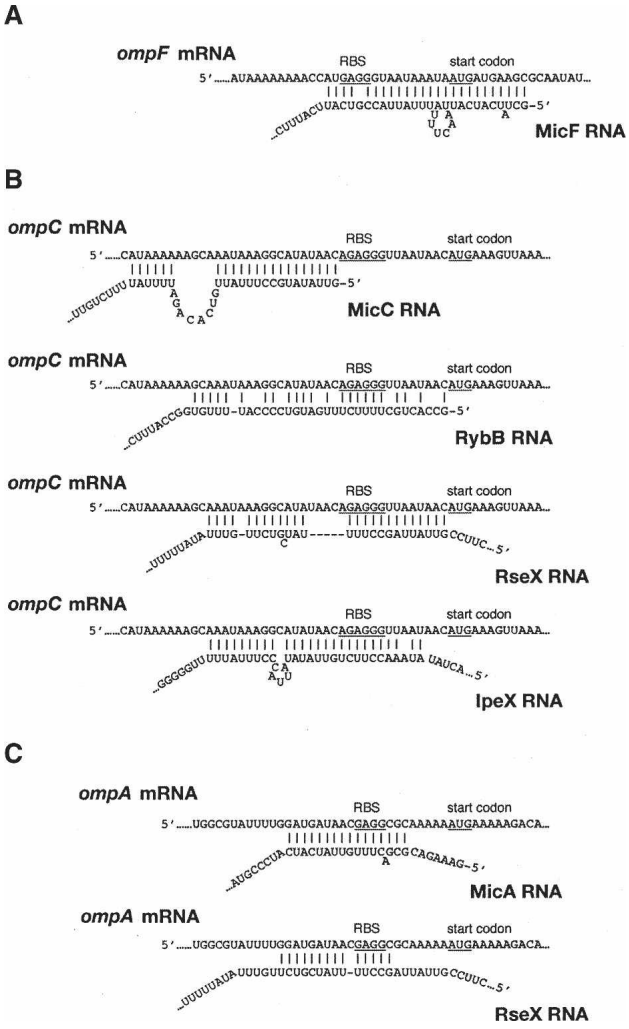


**Figure 1.** Possible base-pairing interactions between the MicF, MicC, MicA, RseX, and IpeX RNAs and the *ompF* (*A*), *ompC* (*B*), and *ompA* (*C*) mRNAs. Ribosome-binding sites (RBS) and start codons are underlined for the mRNAs. Dashes in sRNA sequences correspond to gaps in the base-pairing with the mRNA.

### More $\sigma^E$-regulated sRNAs

As noted above, the genes regulated by $\sigma^E$ are critically important for the proper folding and insertion of outer membrane porins. When the regulatory system for $\sigma^E$ is abrogated—for example, when RseA cannot be cleaved or one of the periplasmic proteases is missing—cells have reduced viability unless fewer outer membrane proteins are synthesized. With whole genome expression analysis to identify genes regulated by overexpression of $\sigma^E$, many genes increased in expression, as expected for those requiring the $\sigma^E$ transcription factor (Rhodius et al. 2006). Most of these were genes involved in the assembly and insertion of β-barrel proteins into the outer membrane (Rhodius et al. 2006; Ruiz et al. 2006). However, some genes were found to decrease in expression, including *ompF*, *ompC*, *ompA*, and *ompX* (Rhodius et al. 2006). From a physiological standpoint, this makes sense; if unassembled outer membrane proteins are present in high enough amounts to induce high levels of $\sigma^E$ activity, reduced synthesis of these proteins should help recovery from this stress. The simplest explanation for the down-regulation is that sRNAs that target these mRNAs are under the control of $\sigma^E$. The observation that the $\sigma^E$-dependent down-regulation of outer membrane porin genes is dependent upon Hfq (V. Rhodius, pers. comm.) is completely consistent with this possibility. $\sigma^E$ regulation of MicA and RybB provides an explanation for the decrease in *ompA* and *ompC* expression; it is likely that still other $\sigma^E$-controlled sRNAs account for the decrease

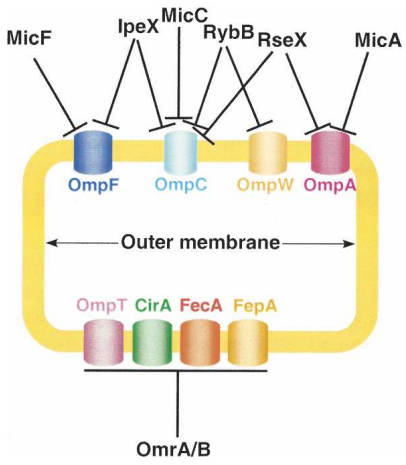


**Figure 2.** Summary of sRNA regulators of outer membrane proteins. sRNAs are shown in black, with their identified targets depicted as outer membrane β-barrel proteins. FepA is more strongly regulated by OmrA than by OmrB (Guillier and Gottesman 2006).

in *ompF* and *ompX* expression. In this light, it is intriguing that Rhodius et al. (2006) detected a $\sigma^E$ promoter upstream and divergent from the *ompX* gene, in a region not encoding any obvious proteins.

## Regulating other outer membrane proteins with sRNAs

While OmpC, OmpF, and OmpA are the most abundant outer membrane porins of *E. coli*, there are other outer membrane proteins that also fold as β-barrels and are capable of acting as channels. These proteins often are only made under specific conditions and are frequently coupled to TonB and energy-dependent inner membrane transporters to aid the efficient entry of specific ligands. New evidence suggests that these outer membrane channel proteins, and other surface molecules, are also subject to post-transcriptional regulation by sRNAs, indicating that it is not simply the high abundance of the major porins that leads to the need for regulation by sRNAs.

### *OmrA and OmrB RNAs*

OmrA and OmrB (previously RygA and RygB or SraE, respectively) were identified in two computational searches for sRNAs (Argaman et al. 2001; Wassarman et al. 2001). They are located in the same *aas-galR* intergenic region, and are transcribed from two distinct, adjacent genes. Both the 88-nt OmrA RNA and the 82-nt OmrB RNA are induced by high osmolarity and regulated by the OmpR response regulator (thus the designation OmpR-regulated sRNAs A and B) (Guillier and Gottesman 2006) and both RNAs bind Hfq (Wassarman et al. 2001; Zhang et al. 2003). Strikingly, their 5′and 3′ ends are almost identical, suggesting that they could have somewhat redundant cellular functions.

Whole genome expression analysis after overexpression of either OmrA or OmrB indicated that these two sRNAs regulate the expression of several genes, the majority negatively. Most of the suggested targets were common to OmrA and OmrB and encode outer membrane proteins. Northern analysis confirmed OmrA- and OmrB-mediated repression for *ompT*, *cirA*, and *fecA*, whose mRNA levels decrease after induction of either sRNA, as well as for *fepA*, whose mRNA levels are much less affected by OmrB than by OmrA (Guillier and Gottesman 2006). *ompT* encodes an outer membrane protease, whereas *cirA*, *fecA*, and *fepA* encode specific gated channels for iron–siderophore complexes; all are members of the β-barrel family of outer membrane proteins.

The mode of action of OmrA and OmrB on their targets is still unclear. Since they bind Hfq and this chaperone is required for the control of OmpT and CirA expression (M. Guillier and S. Gottesman, unpubl.), the sRNAs are predicted to act by pairing. However, the only obvious region of potential base-pairing is between the 5′ conserved regions of OmrA and OmrB and the early translation region of *ompT* mRNA. Preliminary experiments with translational fusions are consistent with a role for this base-pairing in the control of *ompT* expression by OmrA and OmrB in vivo (M. Guillier and S. Gottesman, unpubl.). Only very short regions of potential base-pairing between OmrA or OmrB and *cirA*, *fecA*, or *fepA* mRNAs could be discerned, suggesting that either base-pairing by OmrA and OmrB obey different rules (e.g., shorter regions of pairing) compared to previously reported sRNA–mRNA interactions or that the observed effects of OmrA and OmrB on these targets is indirect.

The induction of OmrA and OmrB expression by the EnvZ/OmpR regulators in response to high osmolarity means that the same signals that favor expression of OmpC, the porin with the smaller pore, also lead to down-regulation of OmpT and some of the iron-regulated gated channel proteins, possibly further limiting entry of toxic molecules.

## Advantages of regulating outer membrane composition with sRNAs

Currently, almost half of the Hfq-binding sRNAs with known targets regulate the expression of outer membrane proteins (Fig. 2). This may reflect the early discovery of MicF and the ease of assaying changes in the levels of the abundant outer membrane proteins, though it seems unlikely that these two factors explain the striking number of outer membrane proteins regulated. Below, we consider some possible reasons for the regulation of outer membrane composition by sRNAs.

### *Need to balance total porin levels*

It is striking that the regulatory sRNAs are frequently proximal to genes for a porin other than the ones they down-regulate. MicF and MicC are encoded opposite the *ompC* and *ompN* genes, respectively; RseX is encoded upstream of the *ompS1* gene; while IpeX is encoded downstream of *nmpC*. This linkage of a porin to a sRNA that reduces the synthesis of other porins might suggest

that the cell needs mechanisms for keeping the total porin load in the outer membrane under strict control. Studies of OmpC and OmpF levels indicate that, although the relative levels of these two proteins change under many different growth conditions, the sum of the two porins is generally constant (Pratt and Silhavy 1995). If there is a need to keep total porin levels relatively constant, it would make sense for specific porins to be linked to a sRNA that would help to make room for that porin. For example, linkage of *micF* to *ompC* would facilitate coevolution of OmpC and a sRNA that could make way for the protein. For the sRNAs linked to minor porins, down-regulation of the major porins may be necessary for full function of the minor porin under the specific conditions when the minor porin is expressed. The presence of the sRNAs with porin genes on cryptic prophages also is consistent with the idea that coevolution of the porins and sRNA regulators is advantageous. However, further study of the roles of the major and minor porins as well as competition between these proteins will be necessary to fully evaluate the significance of the linkage.

If porin load is limiting, it is not clear whether membrane porin occupancy is the problem, or possibly the load on the machinery that assembles the proteins in the outer membrane. The assembly machinery is under the control of $\sigma^E$, and cells become defective for growth under conditions in which the regulatory circuitry is disrupted and unfolded outer membrane proteins accumulate, as well as when $\sigma^E$ is mutated (De Las Penas et al. 1997). The existence of sRNAs to help prevent this is reasonable. Some of the sRNAs discussed here are $\sigma^E$-dependent, consistent with their activation when there is stress on the export machinery. OmrA and OmrB are regulated by OmpR, in parallel to the major outer membrane porins themselves. Thus, these sRNAs and others may allow a subset of porins to be inserted in the membrane by decreasing the levels of others that compete for assembly machinery.

### *Need to respond to the environment*

Implicit in the above schemes for regulating porins is that specific porin proteins are of use to the cell under specific environmental conditions. Nutrients and signaling molecules enter the cells via the porins. However, in some cases the porins may allow the uptake of deleterious compounds and serve as receptors for bacteriophage and toxins such as colicins. For instance, OmpA is the receptor for the T-even bacteriophages (Morona et al. 1984, and references therein) and OmpF and some other outer membrane proteins are receptors for colicins (Fourel et al. 1993). To avoid these stresses, down-regulation of specific membrane proteins in response to specific environmental signals via sRNAs could conceivably minimize the entry of toxic molecules and phage. In addition, by only synthesizing the outer membrane proteins essential under a particular condition, energy and transport capacity is saved for the import of compounds that are most needed by the cell.

Extensive regulation of outer membrane protein synthesis may also be a critical defense against the immune system of hosts. The cell surface is what is seen by the host, and modifying the surface is an important attribute of many successful pathogens. Phase variation, brought about by DNA rearrangements or methylation, changes surface characteristics of many bacteria, presumably helping evasion of the immune system (for review, see van der Woude and Baumler 2004). Down-regulation of outer membrane proteins may play a similar role. In addition to *ompT*, *cirA*, *fecA*, and *fepA*, the OmrA and OmrB RNAs appear to down-regulate genes for fimbriae, consistent with a sRNA role in such a camouflage response (Guillier and Gottesman 2006). InvR, an abundant 80-nt sRNA from pathogenicity island 1 of *Salmonella* was found to down-regulate the level of outer membrane protein OmpD and to be required for full virulence of this organism, supporting a general role for regulation of outer membrane proteins in virulence (V. Pfeiffer, A. Sittka, and J. Vogel, in prep.).

### *Mechanistic considerations*

The arguments that sRNA regulation may be important for balancing porin levels or responding to the environment do not necessarily address why the cell would use sRNAs and post-transcriptional regulation, in place of or in addition to proteins and/or transcriptional regulation. sRNAs that act by base-pairing allow the integration of regulatory signals independently of the target promoter. For the abundant porins, strong promoters and consensus translation signals are likely to be critical for allowing high levels of synthesis under optimum conditions. Such requirements for high expression might necessitate regulation at a post-transcriptional level. Based on whole genome expression analysis, the mRNA levels of *ompA*, *ompC*, and *ompF* grown in rich medium without any special induction are among the highest 100 mRNA signals (e.g., see Massé et al. 2005), along with the ribosomal protein messages, which are also subject to translational control. Possibly for such abundant messages, post-transcriptional regulation is necessary, in addition to any transcriptional regulation, particularly when the level of expression of the protein must be drastically reduced. This argument, however, is less compelling for some of the other proteins such as OmpT and CirA, which are less well expressed. For OmpA, a relatively stable mRNA clearly also contributes to high levels of protein synthesis (Emory and Belasco 1990). However, a survey of the mRNA half-lives of the porins and outer membrane channels does not necessarily support a longer mRNA half-life as the major characteristic of these genes (Bernstein et al. 2004).

The fact that all outer membrane proteins have signal sequences and must translocate across the inner membrane may also render these mRNAs particularly susceptible to post-transcriptional regulation. This argument is supported by the finding that SgrS RNA repression of *ptsG*, which encodes an inner membrane protein, requires membrane targeting (Kawamoto et al. 2005). SgrS

repression of *ptsG* is no longer observed when the sequences encoding the first two transmembrane domains of the PtsG protein are deleted, but is restored by heterologous transmembrane sequences. It is presumed that reduction of the second rounds of translation brought about translocation of the nascent peptide and increases the accessibility of the *ptsG* mRNA to base-pairing with the SgrS RNA. Whether the mRNAs encoding outer membrane proteins are similarly susceptible to regulation by sRNAs because of accessibility of target sequences during translocation of the proteins remains to be determined.

One advantage of sRNA regulators frequently put forth is that the sRNAs are efficient. Because their synthesis is rapid and requires less energy than proteins, sRNA regulators may be more economical under stressful conditions. Another possible advantage of an sRNA over a protein in regulating mRNA stability or translation could be faster recovery once the stress signal disappears and synthesis stops. RyhB base-pairing with its target mRNAs was shown to result in degradation of both the target and the sRNA (Massé et al. 2003). This concomitant degradation of the sRNA means lower levels of the RNA regulator will be present once the inducing signal goes away and transcription of the sRNA is turned off. It is conceivable that this down-regulation of the RNA regulator will allow for quicker adaptation to an environment where the signal for sRNA synthesis is absent (see Lenz et al. 2004).

## Perspectives

Some of the proposals we make here for the physiological roles of sRNAs in regulating outer membrane proteins can be tested experimentally, and presumably will be in the coming years. Identification of all the targets of MicF, MicA, or MicC, for instance, will contribute to our understanding of their function. Are all of their targets outer membrane proteins, or are other pathways also modulated? Homology searches demonstrate that these sRNAs are generally conserved in other Enterobacterial species. Are functional homologs present in other Gram-negative bacteria? Do the bacteria that regulate outer membrane proteins in this way share common growth environments? As the functions of more sRNAs are elucidated, how many others will be found to regulate outer membrane protein synthesis? Conversely, how many outer membrane proteins are subject to known or potential sRNA regulation, and what are the characteristics of the outer membrane proteins that escape this regulation? We look forward to rapid progress in answering these questions.

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# Modulating the outer membrane with small RNAs

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