Small non-coding RNAs and the bacterial outer membrane

Jörg Vogel and Kai Papenfort

Recent systematic genome searches revealed that bacteria encode a tremendous number of small non-coding RNAs (sRNAs). Whereas most of these molecules remain of unknown function, it has become increasingly clear that many of them will act to modulate gene expression at the post-transcriptional level. Where studied in more detail, sRNAs have often been found to control the expression of outer membrane proteins (OMPs). Enterobacteria such as *Escherichia coli* and *Salmonella* are now known to encode at least eight OMP-regulating sRNAs (InvR, MicA, MicC, MicF, OmrAB, RseX and RybB). These sRNAs exert their functions under a variety of growth and stress conditions, including the σ^E-mediated envelope stress response. An sRNA–OMP network is emerging in which some sRNAs act specifically on a single *omp* mRNA, whereas others control multiple *omp* mRNA targets.

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Introduction

Small non-coding RNAs (sRNAs) occur in all kingdoms of life, and have become increasingly recognized as a novel class of gene expression regulators. The eubacterial sRNAs constitute a structurally diverse class of molecules that are typically of 50–250 nucleotides in length and do not commonly contain expressed open reading frames (ORFs). Small RNAs have been known in bacteria since the early 1970s, but only the success of recent systematic genome-wide searches for these molecules and their genes has led to their full appreciation [1–9]. Beginning in 2001, several screens using various methodologies [10•] have increased the number of known sRNAs expressed from the *Escherichia coli* chromosome to greater than 70. Many of the *E. coli* sRNA genes are conserved in closely related pathogens, such as *Salmonella* and *Yersinia* species [11]. It has been estimated that enterobacterial genomes with an average size ranging from 4–5 Mb might contain 200–300 sRNA genes [12]: a figure approximately equal to 5% of the total number of protein-encoding genes.

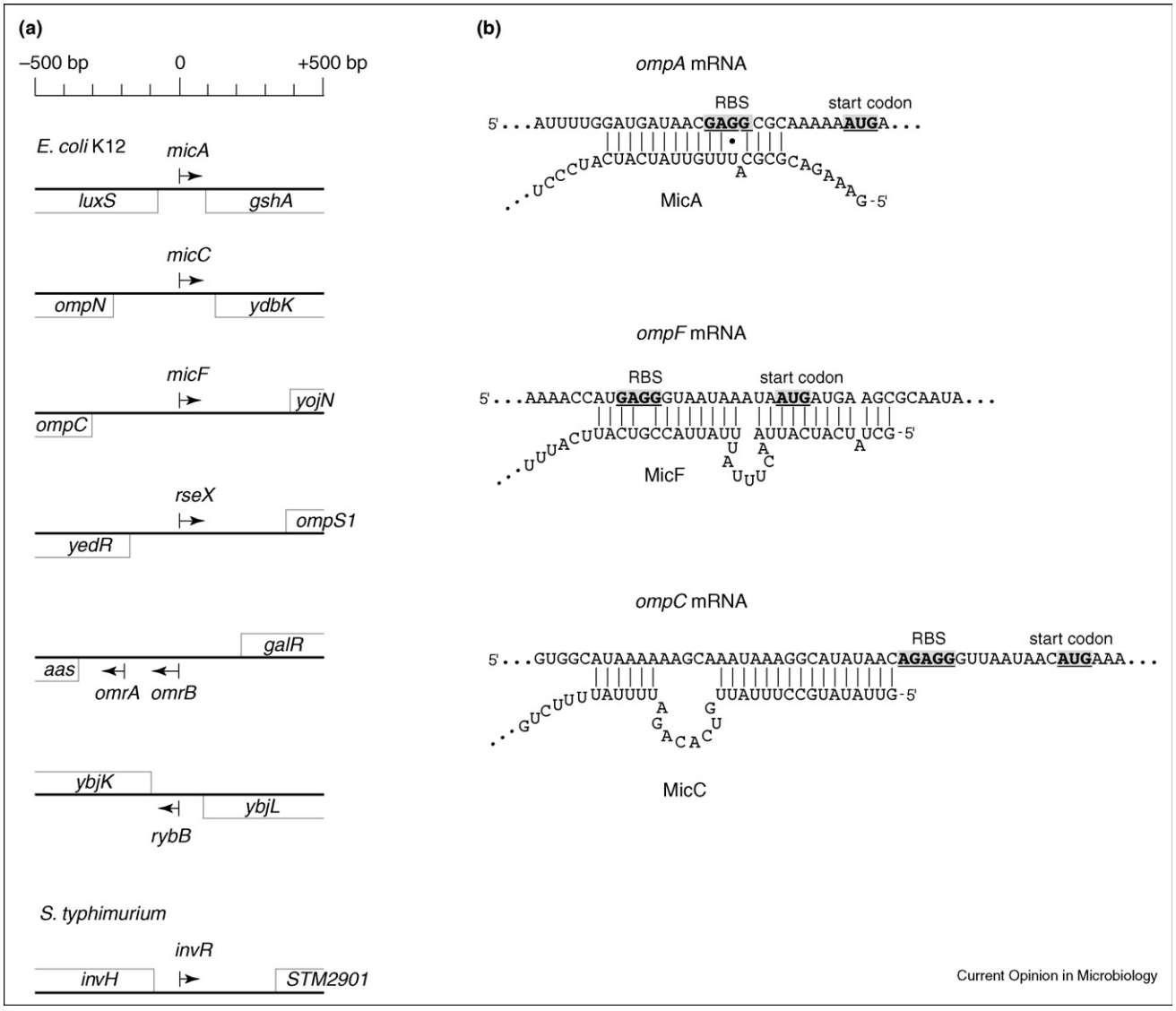
Whereas the search for new bacterial sRNAs is ongoing, ~20 *E. coli* sRNAs have been assigned cellular functions, and often their mode of action has been described (reviewed in [13•–15•]). From these functional studies, it emerges that many sRNAs act as antisense RNAs on *trans*-encoded mRNAs. Unlike the well-studied *cis*-encoded antisense RNAs of plasmids and phages [16], these *trans*-encoded antisense RNAs typically have only short and imperfect complementarity with their target. Base-pairing most often occurs in the 5′ untranslated region (UTR) of the target mRNA, and is aided by the bacterial Sm-like protein, Hfq (host factor for phage Qβ) [17].

The founding member of this class of *trans*-encoded antisense RNAs, MicF RNA of *E. coli*, was discovered more than 20 years ago [18]. The *micF* gene was isolated in a genetic study using its phenotype to repress production of OmpF when present in multiple copies. The 93 nt (nucleotide) MicF RNA forms a ~20 bp imperfect RNA duplex with the translation-initiation region of *ompF* mRNA (Figure 1) [19], in order to negatively regulate expression of this outer membrane protein (OMP) at the post-transcriptional level. Much of this regulation, including the growth conditions that lead to MicF expression, is now well understood and has been reviewed [20,21].

The outer membrane (OM) is a hallmark of Gram-negative bacteria. Together with the peptidoglycan layer and the inner membrane, it forms the bacterial cell envelope. Because of its physical properties, the OM functions as a selective barrier that prevents the entry of many toxic molecules into the cell, and plays a vital role in bacterial survival in diverse environments. However, as membranes are fairly impermeable to hydrophilic solutes, the channels formed by porins such as OmpF facilitate the uptake of nutrients and the excretion of toxic waste-products. Other OMPs that do not function as channels are able to serve as enzymes as well as adhesins. In pathogenic and symbiotic bacteria, the OM represents the bacterial surface that interacts with the eukaryotic host, whilst it also accommodates many proteins that have direct roles in bacterial virulence.

Given the importance of the OM, it does not come as a surprise that the environment-dependent expression of OMPs is extensively coordinated at the level of transcription. However, research in the last two years has shown

Figure 1



OMP-regulatory sRNAs in enterobacteria. **(a)** Genomic location of *E. coli* sRNA genes (*micACF, omrAB, rseX* and *rybB*), and of the pathogenicity island borne *invR* gene in *Salmonella enterica* serovar Typhimurium (+ strand genes above line; – strand genes below line). **(b)** Inhibitory RNA duplexes formed by Mic RNAs with the 5′ UTRs of their respective target mRNAs. The interactions shown were biochemically mapped by *in vitro* structure-probing of MicA–*ompA* and MicF–*ompF* complexes [19,24•,25•], or are supported by the successful introduction of compensatory base-pair changes in the case of MicC–*ompC* mRNA [21]. The AUG start codon and the ribosome binding site (RBS) of the *omp* mRNAs are highlighted.

that aside from MicF, enterobacteria use many additional small RNAs in order to fine-tune the OM composition at the post-transcriptional level. These sRNAs are the subject of this review.

MicC regulates the major porin, OmpC

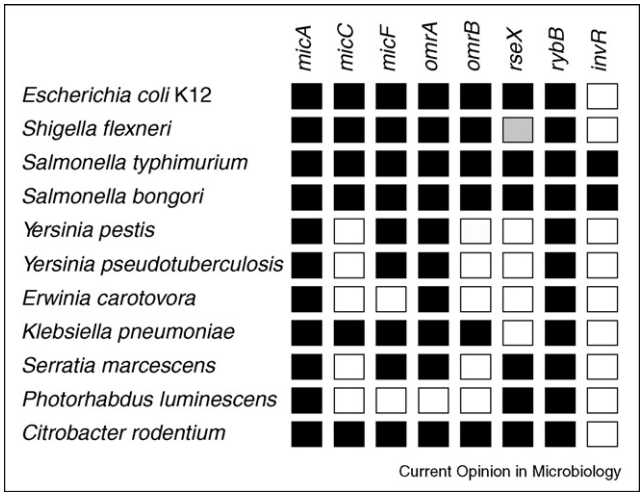
The OmpC and OmpF porins are amongst the most abundant proteins that are translocated to the OM. These proteins span the OM with amphiphatic antiparallel β-strands that adopt a barrel-like conformation, thereby forming a channel. Of the two, OmpC forms the smaller pore, and plays the predominant role under conditions where nutrients, as well as toxins are abundant, whereas the wider OmpF pore is thought to be important under conditions of limiting nutrients and of low toxin levels. The differential expression of these two pore proteins underlies a complex regulation at the transcriptional level [22].

The post-transcriptional repression of OmpF (by MicF RNA) has recently been matched for OmpC through the discovery of MicC [21]. This 109 nt RNA is encoded by a

free-standing gene in the *ompN–ydbK* intergenic region (IGR); hence, a genomic location that is unlinked to *ompC* (Figure 1). Notably, a sRNA gene (IS063) was previously predicted in this IGR in a biocomputational search for 'orphan' transcription signals in the *E. coli* genome [4]. BlastN searches of the *micC* sequence revealed a partial complementarity with the *ompC* 5′ UTR (Figure 1), indicating an inhibitory MicC–*ompC* mRNA interaction [21]. In line with this prediction, MicC overexpression drastically reduced OmpC levels, whereas a *micC* deletion strain exhibits OmpC levels that are twofold higher than the wild type. Further evidence for a ~20 bp MicC–*ompC* mRNA interaction *in vivo* was obtained by the successful introduction of compensatory base-pair changes in the two RNAs. Finally, it was demonstrated that MicC inhibits the binding of 30S ribosome to *ompC* mRNA [21], suggesting that MicC prevents translation initiation.

The *micC* gene is well-conserved among enterobacteria (Figure 2), and its expression is highly regulated. Intriguingly, MicC and MicF expression profiles from a variety of growth conditions showed the two RNAs to accumulate in almost a mutually exclusive fashion [21]. Hence, the discovery of MicC adds another layer to the counter-regulated expression of the OmpC and OmpF porins.

Figure 2



Conservation of genes that encode OMP-regulatory sRNAs in selected Enterobacteriaceae. Presence or absence of a sRNA gene is indicated by filled or open boxes, respectively. The grey box indicates that a *rseX* gene is found in *Shigella dysenteriae* but not in *Shigella flexneri*. Information was collected from [11,21,24•,29•,40,41], and using BlastN searches (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of the following genome sequences (accession numbers are given in parentheses): *E. coli* K12 (NC_000913), *Shigella flexneri* 2a strain 301 (NC_004337), *Salmonella typhimurium* LT2 (NC_003197), *Yersinia pestis* CO92 (NC_003143), *Yersinia pseudotuberculosis* IP 32953 (NC_006155), *Erwinia carotovora* subsp. atroseptica (NC_004547) and *Photorhabdus luminescens* subsp. laumondii (NC_005126). BlastN searches were also performed on the unfinished genome sequences of *Klebsiella pneumoniae* strain Kp342 (available at http://www.tigr.org), and of *Serratia marcescens* strain Db11, *Salmonella bongori* strain 12419 and *Citrobacter rodentium* strain ICC168 (available at http://www.sanger.ac.uk).

Growth rate-dependent control of OmpA expression by MicA RNA

OmpA belongs to a class of proteins that is highly conserved among enterobacteria, it occurs at ~100 000 copies per cell, and it is thought to anchor the OM to the murein layer of the periplasmic space. The *ompA* mRNA is abundant and long-lived, and has long served as a model to study RNA processing and decay. It was early noted that *ompA* mRNA stability varied greatly depending on the growth rate: specifically, this mRNA becomes destabilized at the onset of stationary phase [23]. Over the years, several models were invoked to explain this regulation, including a growth-dependent abundance of two factors, RNase E and Hfq, which were shown to affect *ompA* mRNA decay. However, none of these models was fully consistent, and was often in conflict with reports by others, as summarized in [24•,25•,26].

The recent discovery of the MicA RNA (or SraD) sheds new light on this issue. MicA was first identified in a global *E. coli* sRNA screen, and observed to accumulate as a 70 nt transcript when cells ceased growth [1]. Two groups have now demonstrated that MicA accounts for much of the stationary phase specific instability of *ompA* mRNA [24•,25•]. In wild type cells, MicA levels inversely correlate with *ompA* mRNA levels during growth. However, the stationary phase specific decrease of *ompA* mRNA levels is abrogated upon *micA* deletion. Furthermore, overexpression of MicA results in depletion of OmpA protein. The underlying molecular mechanism is similar to that of MicC and MicF. A 17 nt stretch of RNA located in the MicA 5′ sequence forms a nearly perfect RNA duplex with the *ompA* 5′ UTR (Figure 1), and this interaction is highly conserved in enterobacteria [24•]. Formation of this duplex was demonstrated *in vitro* by footprint analyses of MicA and *ompA* leader-mRNA [24•,25•], and *in vivo* by the successful restoration of repression when compensatory base-pair changes were introduced in the two RNAs [24•]. Finally, toeprint experiments showed that MicA prevented ribosome binding of the *ompA* mRNA [24•].

Does MicA function contradict the previously observed roles of Hfq and RNase E in *ompA* mRNA decay? Rather the opposite, as MicA strongly requires Hfq, both for its own intracellular stability and for annealing to *ompA* mRNA [24•,25•]. Furthermore, it is probable that by masking the *ompA* ribosome binding site, MicA accelerates the RNase E-dependent decay of this mRNA, similar to what has recently been demonstrated for other Hfq-dependent *E. coli* sRNAs [27•,28•]. In as such, MicA provides a growth rate-specific factor for *ompA* mRNA decay.

Two homologous RNAs, OmrA and OmrB, control multiple OMPs

Recent genome searches revealed the existence of two sRNA genes, *omrA* and *omrB* sRNAs, in the ~600 bp intergenic region between *aas* and *galR* (these sRNA genes were first denoted *rygA*/*sraE* and *rygB* [1,2]). Intriguingly, the two sRNAs are of similar length, and are almost identical in their 5′ and 3′ regions, respectively, with these terminal sequences being highly conserved in other bacteria. The 88 nt OmrA RNA accumulates in late stationary phase [1], whereas the 82 nt OmrB RNA is transiently expressed in early stationary phase [5]. Using microarrays to monitor the effects of OmrAB overexpression on global transcript changes, at least four target mRNAs have been identified [29•], all of which encode OMPs (OmpT, CirA, FecA and FepA) and are negatively regulated by either sRNA. Time-course experiments with inducible OmrAB plasmids strongly suggest that the sRNAs accelerate the decay of these target mRNAs. The authors also predicted base-pairing between OmrAB and *ompT* mRNA. Interestingly, much of this interaction would occur right downstream of the *ompT* start codon (i.e. in the leader peptide coding region) and it is currently unclear whether this interaction blocks ribosome entry or directly facilitates *ompT* mRNA decay. We expect that further studies of the OmrAB interactions with their targets will tell us much about how sRNAs co-regulate multiple mRNAs.

Suppressor function of RseX RNA under extracytoplasmic stress

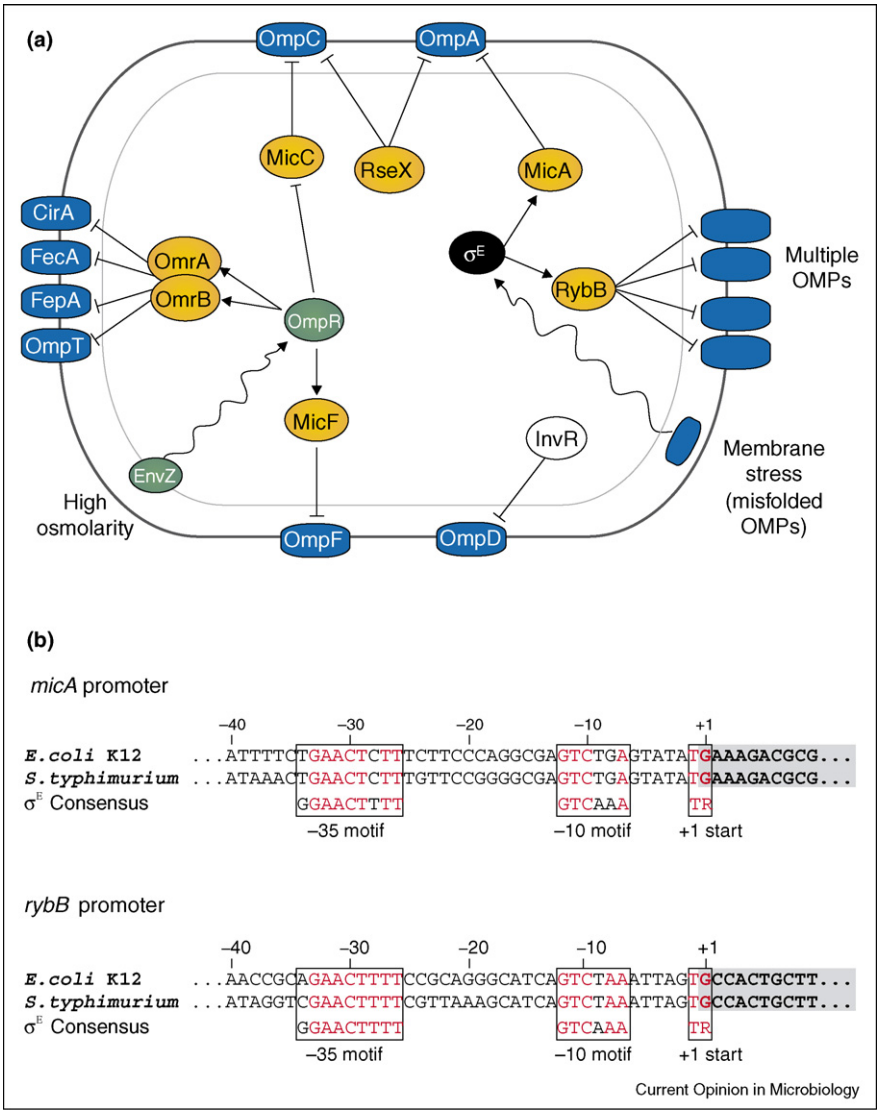
Extracytoplasmic or membrane stress as caused by, for example, the accumulation of misfolded proteins in the periplasm, triggers a global response that is mediated by the alternative sigma factor, σ^E. A key player in the σ^E induction cascade is the protease, RseP, which participates in the release of active σ^E from its membrane-bound precursor complex. The *rseP* gene is essential in *E. coli*. However, a recent screen for multicopy suppressors that could bypass *rseP* resulted in a sRNA surprise: a 170 bp non-coding fragment derived from the *yedR*–*ompS1* IGR was observed as enabling the survival of cells depleted for RseP [30•]. Notably, a sRNA gene (IS096) had previously been predicted in the same IGR [4], although in both studies the chromosomal copy of this gene (now denoted *rseX*) has so far failed to show any detectable transcript under standard growth conditions. However, if *rseX* is carried on a multicopy plasmid, it yields an abundant ~90 nt RNA [30•]. The authors used a novel strategy to capture possible RseX target mRNAs [30•,31]. Synthetic RseX RNA was biotinylated, bound to streptavidin-coated magnetic beads, and incubated with extracted cellular mRNAs. The bound mRNA fraction was then hybridized to whole-genome microarrays, revealing the *ompA* and *ompC* mRNAs as prominent RseX binding partners. The 5′ UTRs of both mRNAs contain regions of complementarity to RseX, and formation of an RseX–*ompA* mRNA complex was observed *in vitro*. Genetic experiments also strongly suggest that *ompA* and *ompC* are indeed the main targets of RseX, because an *ompA*/*ompC* double deletion eliminates the essentiality of RseP protease. Hence, RseX most probably exerts its RseP-bypass function by repressing OmpAC before misfolded intermediates of these proteins accumulate to levels that could be toxic in the absence of an intact σ^E response.

An emerging sRNA–OMP network to fine-tune the OM composition

With the recent discovery of new sRNAs that are involved in OMP regulation, a network is emerging in which some sRNAs act specifically on a single *omp* mRNA (MicA and MicF), whereas others have multiple targets (MicC, OmrA, OmrB and RseX). Likewise, the very same *omp* mRNA could be subject to regulation by more than one sRNA (e.g. *ompC* is regulated by both MicC and RseX; Figure 3). Work in progress in our, and several other laboratories, however, indicates that this is only the tip of the iceberg. We have been performing a large-scale target screen for >20 *Salmonella* sRNAs, most of which are conserved among enterobacteria (K Papenfort and J Vogel, unpublished). The results of this screen strongly indicate the existence of at least three additional sRNAs that target single or multiple *omp* mRNAs. As to multiple target-regulation, the highly conserved RybB RNA, which strongly accumulates in stationary phase [2,5], was found to downregulate more than eight mRNAs that collectively encode OMPs. We have also identified an ~80 nt sRNA, InvR, that is expressed from the *Salmonella* pathogenicity island 1 — the virulence gene region that facilitates *Salmonella* invasion of eukaryotic host cells (Figure 1). The main target of InvR appears to be *ompD* mRNA, encoding one of the most abundant *Salmonella* OMPs (V Pfeiffer and J Vogel, unpublished).

It should be noted that for few of the sRNAs covered here, deletion strains have yielded measurable accumulation of their target OMPs, not to mention their phenotypes. However, the complex regulation of their genes (see below), and the significant overlap in target regulation, might indicate redundancy or even synergy of action. Redundancy of regulatory RNAs is not unheard of: for example, four to five highly homologous sRNAs act in parallel to control a master transcription factor of the quorum sensing cascade in certain *Vibrio* species [32]. Repression of the target mRNA is only abrogated in strains deleted for all of these sRNAs. In addition, growth under standard laboratory conditions does not reflect the often harsh conditions bacteria face when in their natural habitat. For example, a *Salmonella invR* deletion strain, which shows no growth disadvantage in standard media, is compromised for virulence at the early stage of infection in streptomycin-treated mice (A Müller, WD Hardt and J Vogel, unpublished).

Figure 3



An emerging network of sRNAs that control outer membrane protein expression in enterobacteria. **(a)** Regulatory sRNAs are shown in yellow circles within a schematic drawing of an *E. coli* cell. InvR RNA, which is *Salmonella*-specific, is shown as an open circle. The thick black line indicates the outer membrane, whereas the thin grey line indicates the inner membrane. See text for more details on the OMP targets, and the input signals of the network. **(b)** The *micA* and *rybB* promoters contain σ^E consensus motifs previously identified in *E. coli* and *Salmonella* [34,35]. The 40 bp region upstream of the transcription start site (+1), as well as the first 10 nucleotides of the *micA* and *rybB* coding regions (in bold) are shown. Positions that match the residues of the σ^E consensus motifs are shown in red.

σ^E and a two-component system feed the sRNA–OMP network

Networks make the most sense if each player can be pulled by individual strings, so to speak, thus enabling the integration of multiple input signals. In terms of the network described above, some of the sRNAs that regulate the same OMPs are part of different regulons (Figure 3). The EnvZ–OmpR two-component system, known to regulate major porin gene expression in response to high osmolarity, has been implicated in the differential expression of MicC and MicF [21,33]. OmpR-dependent transcription was also observed for *omrAB*, in agreement with predicted OmpR binding sites in the promoter regions of these genes [29•]. That some OMP-regulating sRNAs accumulate in stationary phase also hinted at the involvement of a more general factor: the membrane stress sigma factor, σ^E. Recently, σ^E promoter consensus-motifs have been identified in *E. coli* and *Salmonella* [34,35], and these motifs are prominent in the *micA* and *rybB* promoter regions (Figure 3). The σ^E-dependent transcription of these two sRNA genes has been shown in *Salmonella* (K Papenfort and J Vogel,

unpublished) and by several laboratories in *E. coli* [36••] (K Thompson *et al.* and K Udekwu *et al.*, personal communication), strongly suggesting that these sRNAs counteract membrane stress.

Conclusions and perspective

Small RNA-mediated control of OMP expression, which started with the serendipitous finding of MicF RNA two decades ago, has become an exciting field of research. In many ways, MicF laid the grounding for our current understanding of how bacterial sRNAs modulate the expression of *trans*-encoded target mRNAs. That OMPs and their mRNAs are usually abundant has been an added advantage of identifying these as prominent sRNA targets.

Currently, about a third of the *E. coli* sRNAs with known cellular functions are involved in OMP regulation. Considering that perhaps in the range of 200–300 enterobacterial sRNAs still await functional characterization, one can safely assume that the sRNA–OMP network will continue to grow. However, even where targets are already known, much remains to be learned as to exactly how regulation occurs. It has been puzzling that overexpression of some sRNAs (e.g. MicA) leads to nearly complete depletion of their targets. However, when the target region is fused to a reporter gene such as *lacZ*, only partial regulation is observed [24•]. One explanation for this phenomenon could be that the reporter mRNA is not correctly localized because, in the reporter construct, the OMP signal peptide sequence is incomplete. Intriguingly, it was recently shown that membrane localization of *ptsG* mRNA, encoding an inner membrane protein, is key to regulation by the small RNA, SgrS [37•]. Thus, it will be exciting to see whether such spatial constraints also apply to OMP-encoding target mRNAs.

Assaying the emerging sRNA–OMP network in pathogenic bacteria might give us valuable hints as to why these tiny genes are so conserved (Figure 2). Many OMPs play vital roles for survival outside an animal host, but as a surface-exposed epitope, they are also quickly recognized by the host's immune system upon infection. Even within a host, expression of a given OMP can be a double-edged sword. For example, OmpA of *E. coli* is required to cross the blood barrier, but it is also a target for neutrophil elastase, which will kill wild type *E. coli* but not $OmpA^-$ cells (reviewed in [38]). Yet another detrimental threat to bacteria has not been addressed. Because bacteriophages often use OMPs as receptors for docking (see references in [39]), as bacteria are under phage attack, a rapid shut-off of receptor expression through post-transcriptional control might be a matter of survival. In summary, Gram-negative bacteria often need to quickly adjust the composition of their OM, and we expect regulatory small RNAs to have crucial roles in these adaptation processes.

Update

A first paper describing the σ^E-dependent transcription of the *E. coli micA* and *rybB* genes, as well as the OMP-regulatory function of *E. coli* RybB sRNA, has been published [36••].

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