UNIVERSIDAD D SEVILLA

Genetic and molecular analysis of small regulatory RNAs in *Salmonella enterica*

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Genetic and molecular analysis of small regulatory RNAs in *Salmonella enterica*

Memoria presentada para aspirar al grado de Doctor en Biología

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Directores de la tesis

A los que creyeron en mí y, sobre todo, a los que no

"Success is not final, failure is not fatal: it is the courage to continue that counts."

Winston Churchill

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Introduction

"*Reality is merely an illusion,*
albeit a very persistent one."

Albert Einstein

1. Salmonella

Salmonella is a motile gram-negative bacillus of the *Enterobacteriacea* family. Molecular phylogeny divides *Salmonella* genus in three species: *Salmonella enterica*, *Salmonella bongori* y *Salmonella subterranea* [7, 8]. *Salmonella enterica* is further subdivided into seven subspecies: I, II, IIIa, IIIb, IV, VI y VII. Members of each subspecies are classified in serovars, depending on their antigenic properties.

Salmonella is a pathogen with a broad range of hosts. It most often causes gastroenteritis by proliferating in the intestinal mucosa. Some *Salmonella* serovars are able to produce systemic infection in specific hosts, (*i.e.* typhoid or paratyphoid fever in human). Systemic disease occurs because of bacterial multiplication and dissemination through the lymphoid system, after crossing the intestinal epithelium [9].

Phylogenetically, the *Salmonella* genus is closely related to genera *Escherichia*, *Shigella* and *Citrobacter*. The overall structure of the *Salmonella* genome is highly similar to that of *Escherichia coli* [10, 11]. The average DNA sequence homology between the genomes of non-pathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium is in the order of 80% [12, 13]. Furthermore, *Salmonella* contains specific regions that are not present in other related genera. Some of these regions harbor genes whose products are involved in virulence; these regions are the so-called "*Salmonella* pathogenicity islands" (SPI) [10, 14-20]. In addition, some members of the genus harbor an extra-chromosomal element required for systemic infection, known as the "virulence plasmid" [21, 22]. The size of this plasmid varies between 50 and 90 Kb, although only a short region (7,8 kb), the *spv* locus (for Salmonella plasmid virulence), is thought to directly contribute to the virulent phenotype [23].

2. RNA regulator molecules

The classical view of gene regulation puts most of the emphasis on the transcriptional step. During the last decade, this view has evolved to

incorporate regulatory elements that bring an additional level of complexity to the regulatory process. Besides transcriptional factors, a plethora of RNA regulators acting at the post-transcriptional level have been appeared as essential component of the cell regulatory circuitry. In bacteria, diverse types of gene regulation involving RNA molecules have been described. This heterogeneous group of regulator molecules includes riboswitches, CRISPR (clustered regularly interspaced short palindromic repeats) RNAs and small non-coding RNAs (sRNAs). Riboswitches are cis-acting elements located at the 5' ends of the mRNA that they regulate. These leader sequences fold into structures prone to conformational changes upon the binding of small effector molecules or as a function of physical parameters. Riboswitches thus sense, and respond to, the availability of nutrients in the cell, temperature changes, etc. CRISPR RNAs constitute a recently discovered group of RNA regulators, which contains short regions of homology to bacteriophage and plasmid sequences. CRISPR RNAs interfere with bacteriophage infection and plasmid conjugation, most likely by targeting the region of sequence homology in the invading DNA through an unknown mechanism. RNA regulators act by various mechanisms most of which directly or indirectly target the function of other RNA molecules (reviewed in [24-26]).

2.1 Small regulatory RNAs

The term "small regulatory RNAs (sRNAs)" describes an heterogeneous family of RNA molecules ranging in size between 50 to 400 nucleotides. They are generally "non-coding", except in special cases ([27, 28]) and relatively stable despite being untranslated. Typically, expression of sRNA is activated in response to environmental changes. Their properties and mechanisms of action can vary broadly (reviewed in [26]).

The first regulatory sRNA was discovered about 30 years ago [6], but until the year 2000 the list of sRNAs remained relatively short. Since then, different computational, biochemical and genetic approaches have been applied to search for and characterize sRNAs and their targets (chapter 2)

[29-33] (reviewed in [26, 34, 35]). As a result, the sRNA list has expanded enormously and today includes about 100 entries in *E. coli* and *Salmonella*.

Regulatory sRNAs can be separated into two classes depending on whether they target proteins or RNA. The most representative members of the first class act by titrating translational regulatory proteins. They do so by molecular mimicry: their structures resembling those found in the translation initiation regions of the mRNAs regulated by their target proteins [36-38]. Other protein-targeting sRNAs act on transcriptional regulators, affecting their activity [39] (reviewed in [40]). However, the largest and most extensively studied class of sRNAs acts through base pairing with mRNAs, affecting translation and/or mRNA stability [41, 42] (reviewed in [43]). In polycistronic transcripts, sRNAs can induce its differential degradation, leading to changes in cistron expression ratios [44-47].

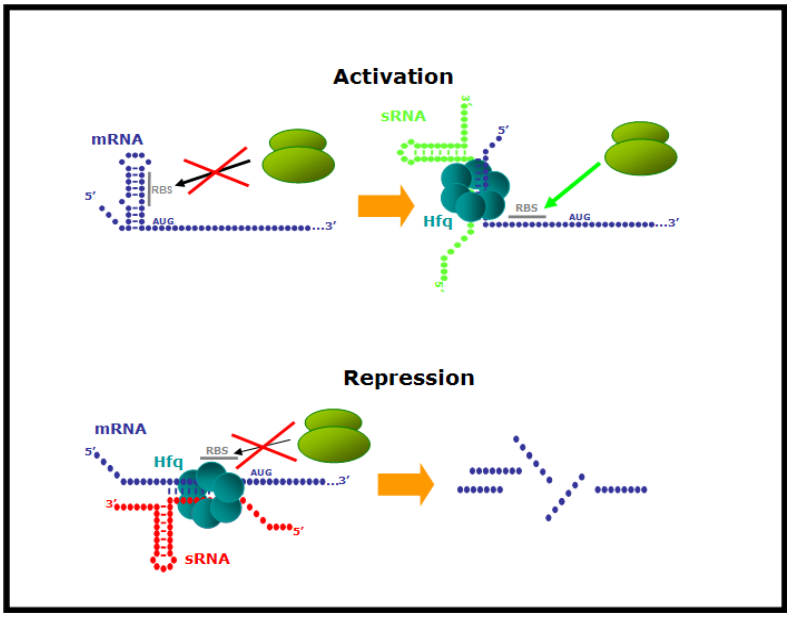
RNA-targeting sRNAs fall into two groups. One group comprises molecules that are encoded at the same locus that they control, but in the opposite strand (often referred to as "*cis*-acting" sRNAs). Typical examples are the sRNAs controlling plasmid copy number and those participating in the regulation of certain bacteriophages (reviewed in [48]). The other group comprises sRNAs that are encoded far from their targets ("*trans*-acting" sRNAs). In general, they base pair with mRNA over short sequence stretches and in most cases require the presence of the RNA chaperon protein Hfq for their function (reviewed in [26]). A single example of an sRNA that can act both in *cis* and in *trans* has been described [49].

Small RNAs can be synthesized as individual transcripts or processed from a larger transcript [50]. Most *trans*-acting sRNAs share some structural features necessary for their function and stability (Figure I1): an Hfq binding region (discussed below), as well as a Rho-independent terminator (a stem-loop and a poly-U tail at the 3' end of the molecule) (chapter 3) [4, 51, 52].

Figure I1. Schematic representation of a sRNA structure. Hfq interaction region is needed (further discussed in next section), as well as a typical Rho-independent terminator stem-loop structure and the poly-U tail at the 3′ end of the molecule.

Small RNAs can act as either positive or negative regulators. Positive regulation occurs when, upon pairing, the sRNA antagonizes the formation of secondary structures that inhibit translation initiation. Folding of the 5′ untranslated region of the mRNA can occlude the ribosome binding site (the Shile-Dalgarno sequence). Pairing by the sRNA promotes a structural change that renders the ribosome binding site accessible for translation initiation [53] (reviewed in [54]) (Figure I2). Nevertheless, the vast majority of sRNAs act as negative regulators. They pair with sequences in the proximity of the Shine-Dalgarno (SD) sequence, blocking the access to the ribosome and/or stimulating mRNA degradation by RNases [41, 55-59] (Figure I2).

Figure I2. Activation or repression of translation by sRNAs in bacteria.



In some cases, the regulatory sRNA pairs at a position far upstream from the Shine-Dalgarno sequence [60-62]. It has been proposed that sRNA can act at this distant site by sequestering ribosome standby site [63] or a translational enhancer element [61] or by triggering degradation of the

mRNA (reviewed in [64]). In other cases, sRNA pairing takes place inside the coding sequence, at the level of the first few codons. Formation of the RNA duplex in this region was shown to prevent translation initiation by affecting ribosome binding and/or eliciting mRNA degradation [1, 57] (chapter 3). Small RNAs can also pair in the spacer regions of polycistronic mRNAs, stimulating the decay of these mRNAs [44-47].

Small RNAs are involved in the regulation of many fundamental physiological processes. These include regulation of iron, sugar and amino acid uptake, response to different stress conditions, membrane biogenesis, plasmid stability and transfer, quorum sensing, control of toxin-antitoxin systems, SOS induction, biofilm formation and regulation of virulence process [65] (reviewed in [66, 67]).

The involvement of sRNAs in these processes is directed toward improving bacterial adaptation to environmental changes. Thus, for example, several sRNAs modulate the protein composition of the outer membrane, the compartment most directly in contact with the environment. All of the major porins and several outer membrane proteins (OMPs) are targets for regulation by one or more sRNAs. The genes encoding these sRNAs are generally regulated at the transcriptional level, and expressed under specific conditions (chapters 2 and 3) [1, 3, 4, 6, 52, 68-80] (reviewed in [72, 81-83]).

Conceivably, adaptation to environmental changes or stress conditions demand rapid regulatory responses. By virtue of their small size and the fact that they are not translated, sRNAs are expected to be operational within instants at the onset of the response [84]. sRNAs also permit to establish a hierarchical level of regulation above transcriptional control. In some cases, translational regulation by the sRNA can bypass transcriptional regulation of the same gene. For instance, ChiX-mediated translational repression of *chiP* mRNA overrides constitutive transcription of *chiP* gene [52].

Finally, an important feature of sRNA function is that these molecules act stoichiometrically, that is, they undergo degradation upon pairing to their target [41]. Since each sRNA molecule will act only once, it follows that maintenance of the regulatory response depends on the continuous expression of the sRNA gene. Clearly this allows a rapid reversal of the response once the conditions leading to sRNA expression are no longer present. The dependence of the response on relative transcription rates of sRNA and mRNA allow to establish mRNA and sRNA expression thresholds above or below whose regulation is no longer exerted [85]. Furthermore, since the regulatory activity of the sRNA often aims at correcting such conditions, the system is particularly suitable for homeostatic regulation (reviewed in [86]). An interesting development regarding this aspect has been the discovery of RNA molecules that mimic the mRNA target of an sRNA and sequester (and promote degradation of) the sRNA, thus blocking its action [52, 76, 87]. Multiple sRNAs, expressed under different conditions, can regulate the same target, allowing the integration of diverse environmental signals into the same regulatory pathway [3, 53, 57, 88].

3. Hfq

Escherichia coli Hfq is a 102 amino acids protein (11.2 kDa) that was first identified as a host factor for RNA phage Qβ RNA replication (also known as host factor 1) [89-91]. The gene coding for Hfq is present in approximately half of all sequenced Gram-positive and Gram-negative genomes, with some bacteria having more than one *hfq* gene [92, 93]. At least one archaeon containing a protein related to Hfq has been reported [94].

Proteins of the Hfq family are thermostable, range in length from 70 to 110 amino acids and organize in an homohexameric structure (Figure I3) [90, 93, 95]. In *E. coli*, Hfq is present at approximately 10000 hexamers per cell, 80% of which are found in the cytoplasmic fraction, associated with ribosomes, with a significant amount of Hfq located in the close proximity to the cytoplasmic membrane [96-98].

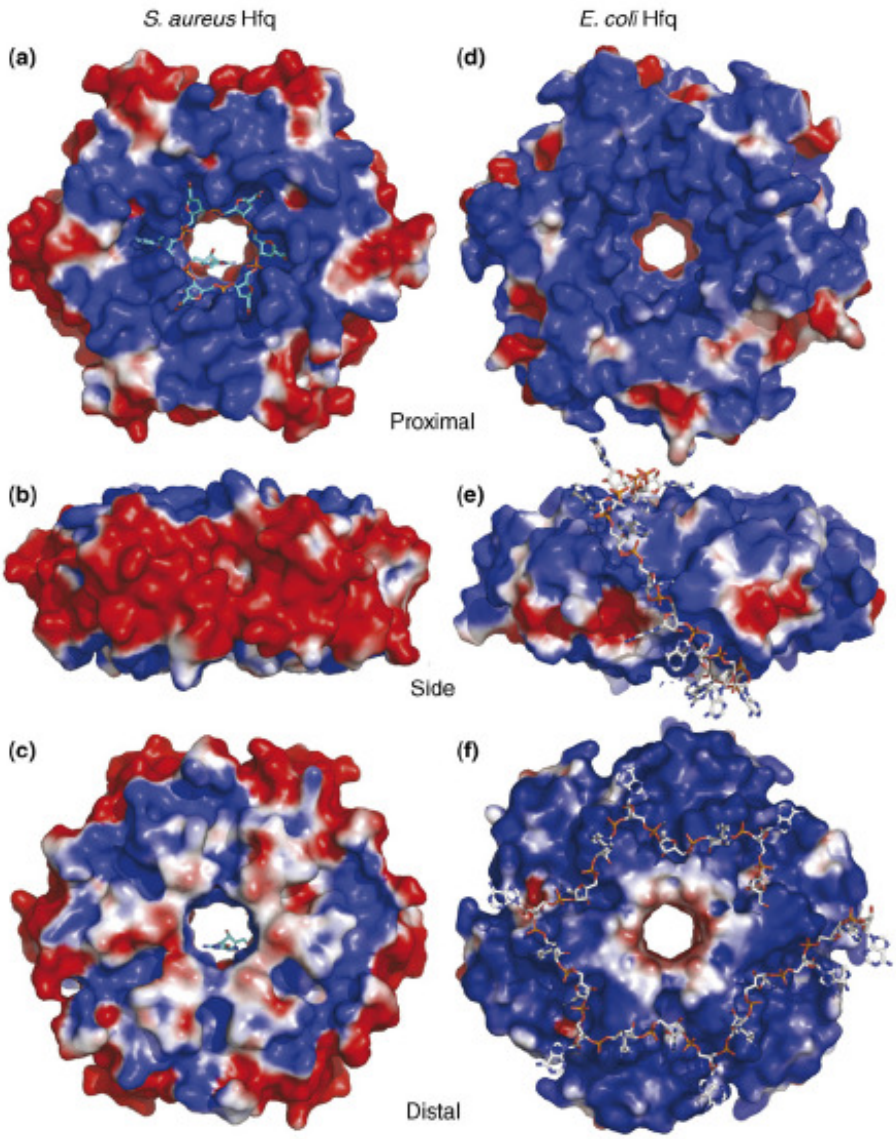


Figure I3. Electrostatic potential energy surfaces of the known and proposed RNA binding sites of the S. aureus and E. coli Hfq proteins. (a–c) Views of the proximal side and distal electrostatic surfaces of S. aureus Hfq, respectively. (d–f) Views of the proximal, side and distal electrostatic surfaces of E. coli Hfq, respectively (blue is electropositive and red is electronegative). The side view of E. coli Hfq includes a plausible RNA binding cleft that would enable A27 to bind to both the Proximal and Distal Sites. The view of the Distal Site of the E. coli Hfq shows a possible binding site for A18 (i.e. a poly(A) tail). The bound RNAs are shown as solid sticks with carbon, nitrogen, oxygen and phosphorous atoms colored white, blue, red and orange, respectively. The electrostatic potential energy surfaces were created by PyMol and the APBS plug-in (DelanoScientific LLC, Palo Alto, CA) [99].

Hfq binds RNA, with higher affinity for poly(A) [100-103] and for short, single-stranded AU-rich stretch, either preceded or followed by a stem-loop structure [104, 105]. Hfq also interacts with proteins. Interactions with ribosomal protein S1, RNA polymerase, PNP, PAP I, Rnase E and Hfq itself has been described (see below) (reviewed in [99]).

3.1. Roles of Hfq protein

Beyond Qβ replication, role of Hfq in cellular processes has been revealed through the study of *hfq* null mutants in different organisms. Early observation of the complex phenotypes of these mutants (reviewed in [106]) were explained by the discovery of the Hfq involvement in the regulatory action of many different sRNAs [107] (reviewed in [66, 99, 108]).

In part, the pleiotropic effects of *hfq* mutations reflect the requirement of Hfq for efficient translation of the *rpoS* gene, which encodes σ^S, an alternative sigma factor expressed under a variety of stress conditions and in stationary phase [109-111] (see next section). At least four sRNAs have been described as regulators of *rpoS* translation (three as activators and one as a repressor) [112-116]. Hfq binds to all four these sRNAs and stimulates their pairing to the 5' untranslated region of *rpoS* mRNA (reviewed in [54, 93, 117]). A further reason for the pleiotropy of Hfq defects relates to σ^E response. In the first paper included in this thesis (chapter 2), we report that Hfq inactivation causes cleavage of anti-σ^E factor RseA, leading to the constitutive induction of the σ^E-dependent envelope stress response. RseA cleavage occurs as a result of the loss of sRNA-mediated negative regulation of porin biogenesis in an *hfq* mutant (chapter 2) [118] (see next section).

Hfq binds both the sRNAs and the mRNA target in the proximity of the pairing region. Such binding stabilizes both pairing partners [33, 93] and facilitate their interaction [95, 119]. This action is likely necessary due to the shortness of the pairing sequences. Hfq binding may orient these sequences in a way more suitable for pairing and/or simply increase the local concentrations of the pairing partners [120-122]. Hfq is also thought to autoregulate its own translation by binding two sites on the *hfq* mRNA, thereby inhibiting the formation of the translational initiation complex [123].

Besides its role in sRNA:mRNA pairing, Hfq directly affects the decay of some mRNAs by binding to their poly(A) tails, stimulating polyadenylation by poly(A) polymerase I (PAP I) and protecting this messenger from polynucleotide phosphorylase (PNP), Rnase II and Rnase E [102, 124-129].

Hfq has also found to copurifies in with PNP and PAP I, two components of the degradosome. An Hfq-PNP-PAP I complex has clear implications for the role of Hfq in regulating the polyadenylation of mRNAs that contain Rho-independent transcription terminators. Hfq could destabilize the terminator stems, which are the direct targets of PAP I and ultimately favor degradation by PNP and Rnase II [129, 130]. Besides PAP I, it has been demonstrated that Hfq stimulates the activity of another protein of the nucleotidyltransferase superfamily: CCA-adding enzyme, which synthesizes the 3'-terminal sequence C-C-A added to all tRNAs [131].

Furthermore, Hfq was reported to form a complex with Rnase E when other components of degradosome (PNP, RhlB, enolase) are not present. Binding of an sRNA to Hfq does not appear necessary for the formation of a stable Hfq-Rnase E complex. Moreover, given that Hfq and other degradosome components (except enolase) bind to the same RNase E domain (C-terminal scaffold), there may be a competition for binding. RNase E-Hfq-sRNA complex results in translational repression and rapid target mRNA degradation [41, 42, 56, 132].

Hfq protein oligomerizes *in vitro*. The protein was shown to form well-ordered fibers that resemble those seen previously in archaeal Sm proteins (SmAPs). *In vivo* formation of this fibrillar structures has not yet determined, and the only physiological function proposed for this Hfq fibers is to constitute a storage form of the protein [133].

Finally, ribosomal protein S1 has been shown to mediate Hfq binding to RNA polymerase, thereby affecting transcription [134, 135].

4. Stress responses in bacteria

Bacteria possess signaling systems that sense specific stimuli associated with changing environmental conditions. Stress signals may activate multiple response pathways to build an integrated and complex response. In *Salmonella*, the different stress responses are controlled by master regulators that coordinate changes in gene expression patterns. Some of these regulation systems involve the activity of alternative sigma factors, which direct the transcription of different sets of genes.

In *Escherichia coli* and *Salmonella* Typhimurium, transcription of housekeeping genes results from the activity of RNA polymerase (RNAP) holoenzyme associated with σ^{70} (σ^{70}, RpoD). However, there exist six additional σ factors that each recognizes a unique set of promoters, often related through a common function. σ^{32} (σ^{H}, RpoH) controls heat shock promoters, σ^{54} (σ^{N}, RpoN) controls mostly promoters for nitrogen assimilation, σ^{S} (σ^{38}, RpoS) turns on stationary-phase (and other) promoters, σ^{F} (σ^{28}, RpoF) is implicated in transcription of genes for flagellum-related functions, σ^{fecI} recognizes promoters involved in iron transport, and σ^{E} (σ^{24}, RpoE) controls responses to extracytoplasmic stresses (reviewed in [136]).

Among those mentioned, σ^{S} is the main sigma factor implicated in the response to stress conditions and, for this reason, it is considered a key master regulator in *Salmonella* adaptation and survival during exposure to environmental insults (reviewed in [137]).

4.1. General stress response. Sigma S

Sigma S is nearly absent in rapidly growing cells, but strongly accumulates in response to a variety of stress conditions. In this case, it partially replaces the housekeeping sigma subunit (σ^{70}) in RNA polymerase and reprograms this enzyme to transcribe sigmaS-dependent genes (up to 10% of total genes) [138]. Sigma S is considered the master regulator of the general stress response as it is essential for the expression of multiples stress resistance genes. Moreover, it should also be considered as second

housekeeping sigma factor with a major impact not only on stress tolerance but also on the entire cell physiology under suboptimal growth conditions. The σ^S network overlaps extensively with other regulons since certain modules belonging to the general stress response can be temporarily recruited by other stress-responsive regulators acting in concert to σ^{70} RNA polymerase. Thus, not only the expression of genes within a regulatory network but also the architecture of the network itself can be the subject of regulation [139] (reviewed in [140]).

RpoS regulation is a complex phenomenon, comprising mechanisms acting at the transcriptional, translational and post-translational levels, all tightly coordinated in response to stress signals. Low levels of carbon, nitrogen or phosphorus, as well as amino acid starvation, trigger RpoS synthesis. Increased RpoS levels during stationary phase are due to increased transcription [141], greater efficiency of translation [112-116] and increased protein stability [142] (Figure I4).

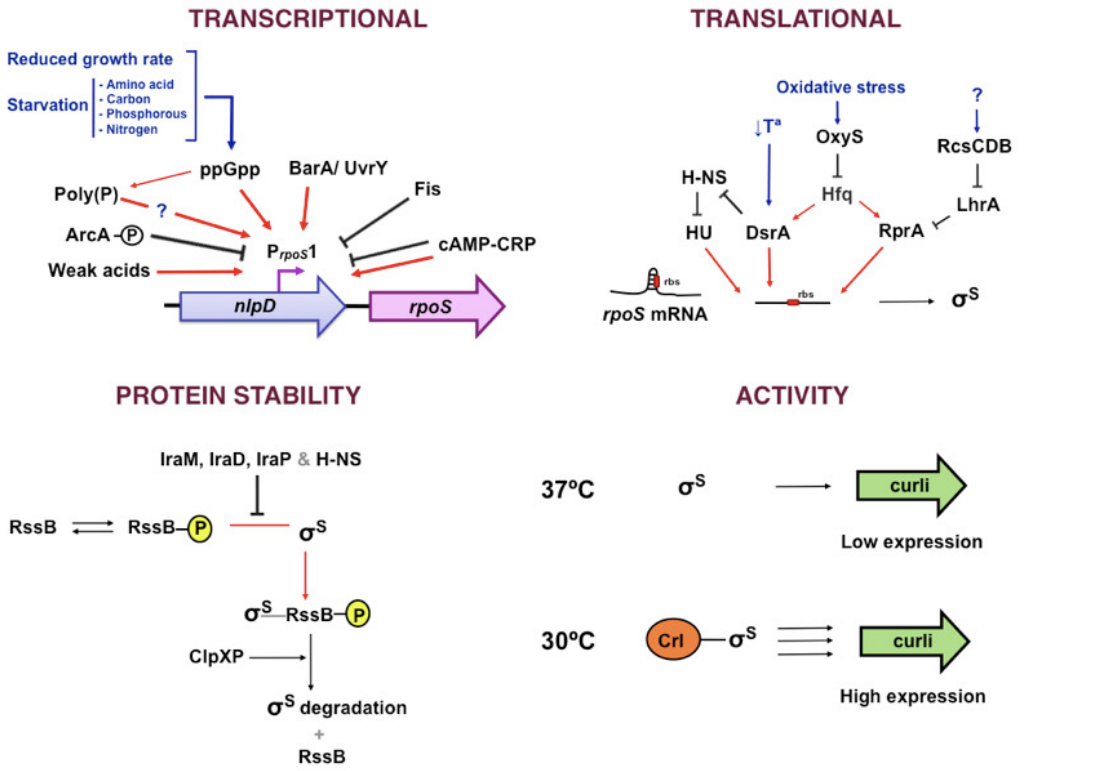


Figure I4. Regulation transcriptional, translational and at the level of protein stability of RpoS in *Escherichia coli* [140].

4.2. Extracytoplasmic stress signaling

The bacterial envelope is involved in essential processes including nutrient transport, respiration, secretion, adhesion, virulence and maintenance of bacterial integrity. In Gram-negative bacteria such as *Salmonella*, the envelope comprises an inner membrane, a periplasmic space that contains the cell wall (peptidoglycan), an outer membrane and bacterial surface structures such as fimbria, pili and flagella. Both membranes lodge a variety porins, lipoproteins, transport proteins and enzymes.

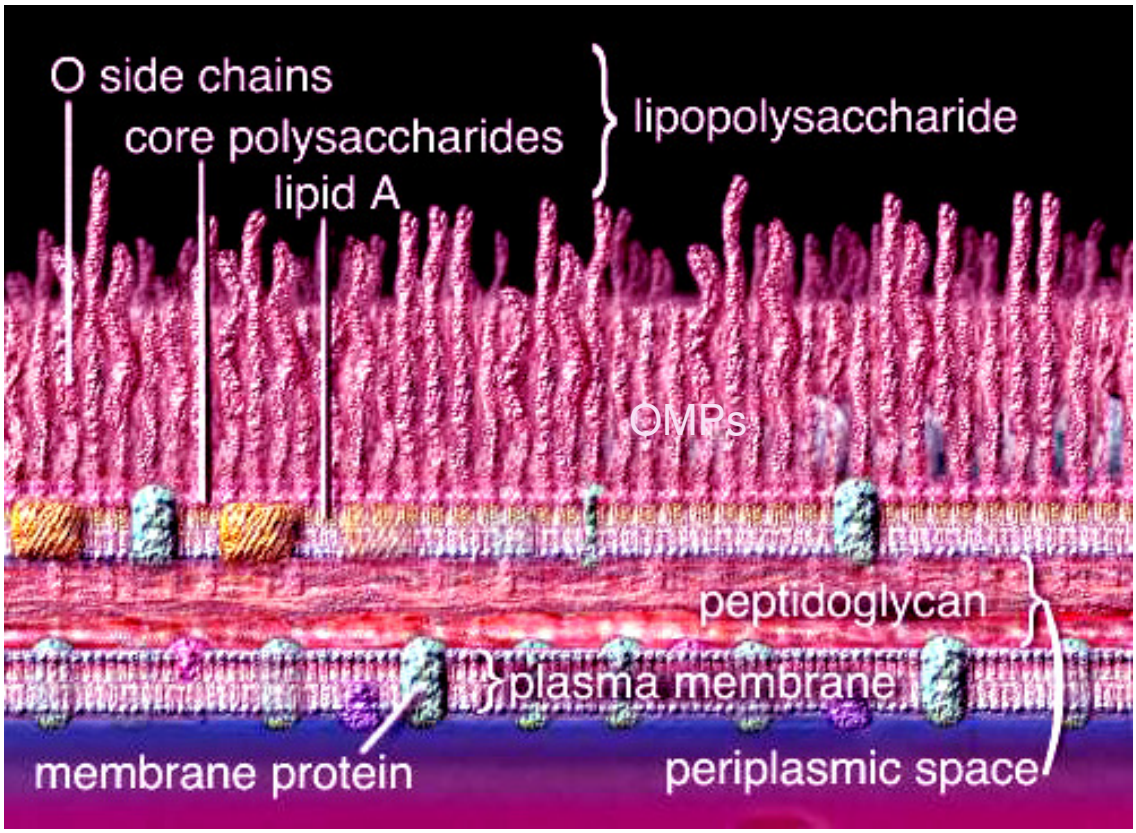


Figure I5. Gram-negative bacterial cell wall structure diagram (modified from [Russell Kightley Media (rkm.com.au)]).

In direct contact with the external medium, the envelope is the initial target of physical (e.g., hyperthermia, osmolarity), chemical (e.g., ethanol, pH, detergent) and biological (e.g., adhesion, infection) insults that may alter envelope components, thus inducing an extracytoplasmic stress response (reviewed in [143]).

The Bae, Cpx, Psp, Rcs, and σ^E pathways constitute the *Salmonella* signaling systems that detect and respond to alterations of the bacterial envelope (reviewed in [2]). The five pathways regulate chaperones, peptidyl-prolyl cis-trans isomerases, periplasmic disulfide isomerases, proteases that are involved in the folding or degradation of misfolded proteins, and also some steps in the biosynthesis of envelope components (reviewed in [144]). Together, these five envelope stress response systems contribute to maintenance and repair of the bacterial envelope. In addition, they modulate key bacterial physiological functions, such as motility, colony and biofilm formation, conjugation, stationary phase adaptation, and virulence (reviewed in [143]).

4.2.1. Envelope stress response. Sigma E

Stress responses have evolved in Gram-negative bacteria to maintain the outer membrane integrity. In *E. coli* and *Salmonella*, conditions that perturb folding of outer membrane proteins (OMPs), trigger a proteolytic cascade that results in the activation of the alternative sigma factor, σ^E [145-147]. In the absence of inducing signals, σ^E is sequestered on the cytoplasmic side of the inner membrane by the antisigma factor RseA. A periplasmic protein, RseB, binds to the periplasmic domain of RseA and enhances the inhibition of σ^E [148, 149]. Upon envelope stress, peptide sequences becoming exposed due to unfolding or misfolding of OMPs bind to the PDZ domain of inner-membrane anchored DegS protease, activating cleavage of the periplasmic domain of RseA [150-154].

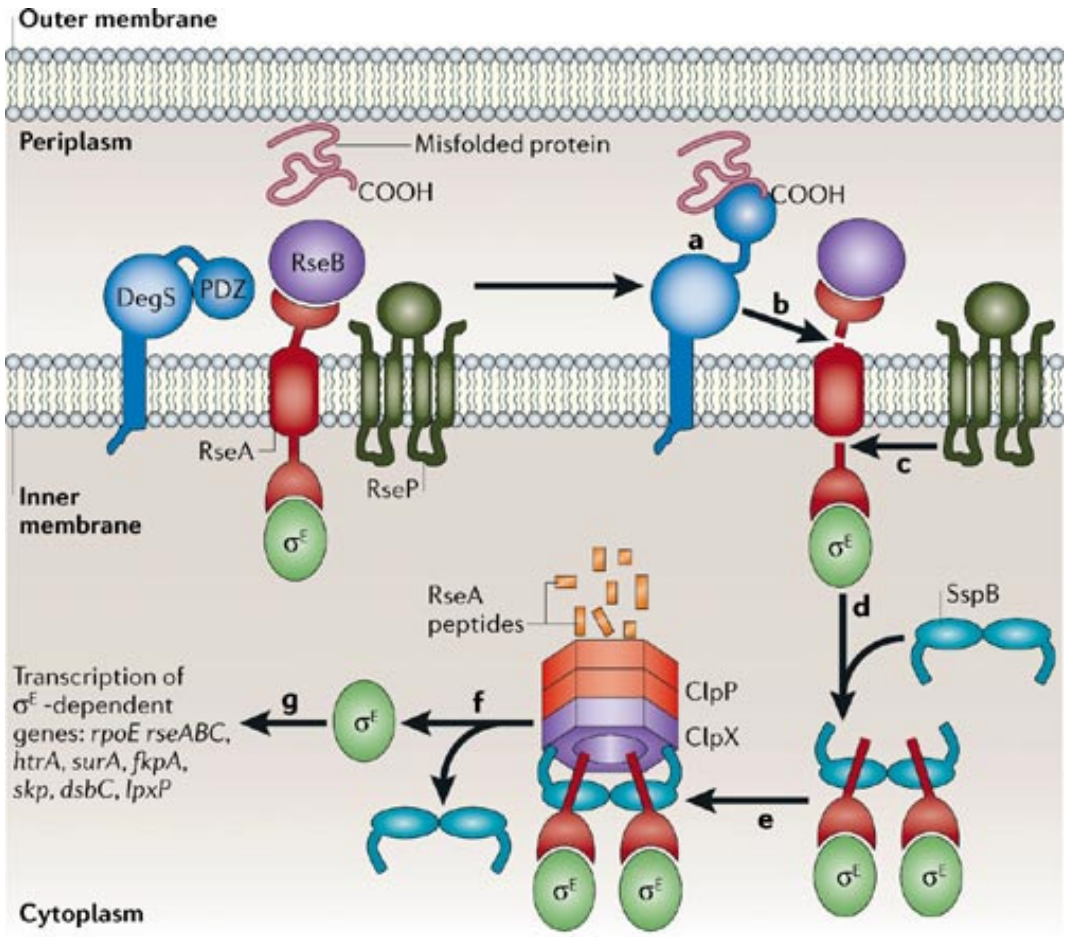


Figure I6. Model for regulation of activity by regulated intramembrane proteolysis (RIP) in *Escherichia coli* [2].

In addition to allosteric regulation of DegS, the proteolytic cascade is also inhibited by the periplasmic protein RseB [155]. RseB binds to the periplasmic domain of RseA and increases its stability by blocking its DegS-mediated cleavage [150, 156]. This suggest that an additional step is required for activation of the response, and RseB must first be removed from RseA for DegS to act [157, 158]. Crystalographic studies reveal that RseB has two domains, one of which closely resembles lipoprotein-binding domains. This observation suggests that RseB may sense disruptions of lipoprotein synthesis either acting in conjunction with the OMP signals or providing a new trigger for pathway activation [159, 160].

RseA cleavage by DegS releases a carboxyl terminal hydrophobic amino acid, creating a substrate for the second protease in the pathway, the membrane-embedded RseP [154, 161]. The fact that RseP cannot degrade RseA independently of the signaling pathway [158, 162, 163], confers robustness to the system and protects against ungranted activation. RseP is held in the inactive state by DegS, RseB, the PDZ domain of RseP, and two regions of RseA [158, 163]. However, a DegS-independent activation of RseA cleavage by RseP during acid stress has been reported, suggesting that low pH may disrupt an inhibitory interaction between RseA and RseP to allow RseA proteolysis without DegS intervention [164]. RseP cleaves RseA in the transmembrane region, releasing its cytoplasmic domain (still bound to σ^E), which is then subject to degradation by cytoplasmic proteases, primarily ClpXP [146, 165, 166]. The C-terminal tail of $RseA_{cyto}$ also contains recognition sequences for the SspB adaptor protein, facilitating proteolysis of $RseA_{cyto}$ by ClpXP (Figure 6) (reviewed in [2, 167]).

Once free in the cytoplasm, σ^E factor associates with RNA polymerase core enzyme activating the transcription of a set of genes encoding proteins related to diverse physiological processes. These include periplasmic proteases and folding factors, proteins involved in cell membrane integrity and in phospholipid and LPS biosynthesis, regulatory proteins, primary metabolism proteins and membrane or periplasmic proteins of unknown function. Furthermore σ^E activates transcription of it own gene along with the *rseABC* operon. The members of the σ^E regulon are thought to help the

bacterium recover from stress conditions. Interestingly, some of the σ^E–regulated genes are critically important for *Salmonella* virulence [148, 149, 168-183].

Concomitant to the activation of several genes, another consequence of the σ^E response activation is the repression of all major OMPs (Figure I7) [168, 170, 174, 179, 181].

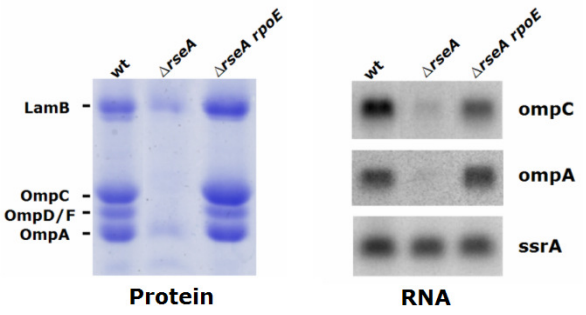


Figure I7. Analysis of OMP protein and RNA patterns in *Salmonella rseA* mutant (constitutively active σ^E response) and *rseA rpoE* double mutant (non-functional σ^E) strains demostrates σ^E–dependent downregulation of OMPs (modified from [4]).

This repression results from the activity of sRNAs whose genes belong to σ^E regulon. MicA, RybB and CyaR sRNAs have been shown to be under positive control of σ^E in *E.coli,* but only the two first are σ^E-dependent in *Salmonella.* The promoters of *micA* and *rybB* genes match the consensus sequence of σ^E promoters, and their activity has been shown to be strictly dependent on σ^E (Figure I8) (chapter 2) [1, 3, 4, 73, 74, 79, 118] (reviewed in [72, 81-83]).

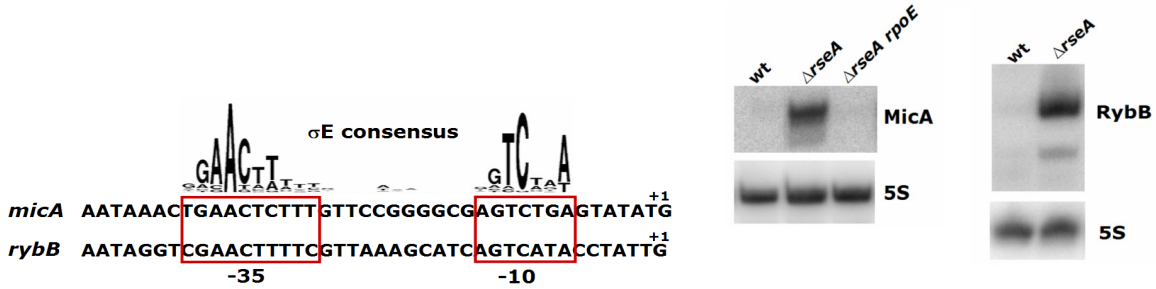


Figure I8. Left: Representation of *micA* and *rybB* promoter sequences and consensus for σ^E-dependent promoters (in the WebLogo format) [181]. **Right:** Northern anlysis shows that presence of MicA and RybB sRNAs depends on σ^E (modified from [4]).

MicA and RybB are responsible for downregulation of major outer membrane proteins in *Salmonella* (Figure I9). The repression is at post-transcriptional level and involves the establishment of a base pair interaction between the sRNA and the *omp* mRNA (chapter 2) [1, 3, 4, 59, 74] (reviewed in [72, 81-83]).

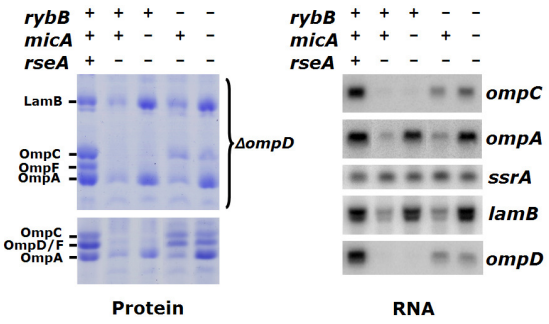


Figure I9. Analysis of OMP protein and RNA patterns in *Salmonella rseA* mutant strains (constitutively active σ^E response). Deletion of either *micA* or *rybB* relieves σ^E-mediated downregulation of OMPs (modified from [4]).

5. RybB

RybB is a ≈80nt sRNA originally identified in a whole genomic search for small RNA in *E. coli* [32]. RybB was subsequently shown to interact with Hfq [33] and to downregulate *ompC* and *ompW* mRNAs in *E. coli* [74, 79]. In *Salmonella enterica*, it has also been shown to downregulate *ompA, ompC, ompD, ompF, ompN, ompS, ompW, fadL* (Figure I10), [1, 3]) and *chiP* mRNAs (chapter 3).

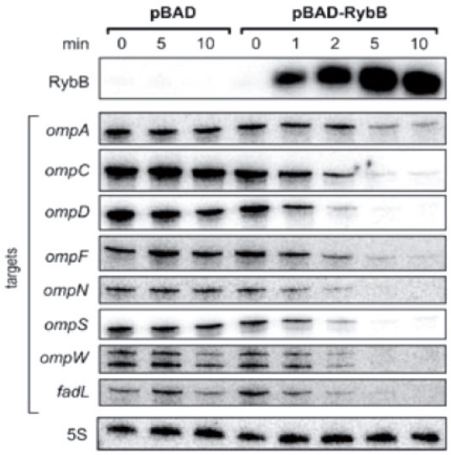


Figure I10. Northern blot validation of RybB-induced target mRNA decay. *Salmonella* carrying either the control pBAD vector or the pBAD-RybB expression vector were arabinose-induced at an OD of 1.5, and total RNA extracted at the time-points indicated above the panels. Northern hybridization with gene-specific probes (indicated to the left) confirmed rapid induction of RybB expression (upper panel), and a concomitant drop in the steady-state levels of eight target mRNAs (*ompA/C/D/F/N/S/W*, and *fadL*) in pBAD-RybB cells. Probing for 5S rRNA confirmed equal RNA loading (lower panel) (modified from [3]).

RybB::mRNA interactions can take place in the 5'-UTR region or inside coding sequence close to the translation start [3]. In the case of *ompN*, RybB can act as translational repressor by sequestering sequences within the mRNA up to the fifth codon [1] (chapter 3).

Some RybB-regulated OMPs are also regulated by other sRNAs in *E.coli* and *Salmonella*. MicF downregulates OmpF [5, 6], MicA downregulates OmpA [59], MicC downregulates OmpC [68] and OmpD [57], and InvR – an SPI-1-encoded sRNA – downregulates OmpD [88]. The fact that a single sRNA can

regulate multiple target and that, in turns, some targets are regulated by more than on sRNA illustrates the relevance of the system for integrating different environmental signals. Because of this pleiotropy, it is conceivable that stoichiometry could play a major role in sRNA-mediated regulation (chapter 3) [52].

6. Role of sRNAs in evolutionary events

Unlike eukaryotes, which evolve mainly through the modification of pre-existing genetic information, bacterial genetic diversity comes in a significant part from the acquisition of sequences through lateral transfer. These lateral transfer events can change the physiology and ecology of bacterial species, conferring selective advantages under certain environmental conditions [184-186] (reviewed in [20, 187-191]).

Thus, for example, gene transfer is thought to have played a major role in the emergence of bacterial pathogens. In particular, the acquisition of so-called pathogenicity islands is considered a key event in the conversion of ancestral extracellular bacteria into intracellular pathogens [192-202] (reviewed in [10, 14-19, 203-207]).

DNA acquisition produce composite genomes characterized by a conserved core interspersed with more recently acquired regions. In pathogens, regulation of virulence functions requires communication between the core and the variable regions, in order to integrate the acquired material into existing regulatory networks.

Bacteria regulate expression of island-encoded genes through general mechanisms or specific pathways. For example, histone-like protein H-NS (requiring also HhA) has been shown to discriminate between the core genome and acquired DNA in *Salmonella*. H-NS-mediated silencing of horizontally acquired DNA prevents undesidered deleterious effects derived from expression of exogenous genes. Occasionally, the relief of such silencing offers a competitive advantage for adaptation [208-213]. Several core-encoded regulators specifically modulate expression of acquired

elements. Furthermore, regulators encoded in acquired regions can affect expression of core genome genes, establishing a cross-talk between foreign and resident genes. Cross-regulation between separate acquired elements has also been described [214-233] (reviewed in [234-236]). sRNAs can participate in this cross-talk. For instance, SPI-1-encoded InvR sRNA represses the synthesis of the core genome-encoded outer membrane porin OmpD [88]. Besides, several sRNAs encoded within genetic islands of *Salmonella* show host-induced expression and have potential roles in virulence [237, 238].

In many cases, DNA acquisition involves the integrative recombination of circular DNA molecules into the host genome. This process is catalyzed by integrases, a class of site-specific recombinases encoded by temperate bacteriophages and plasmids. Integrases introduce staggered cuts at specific sequences on both donor and host DNAs and promote strand exchange and ligation [239, 240]. As a result, the sequences recognized by the integrase are duplicated at each end of the inserted DNA. In some cases such direct repeats are conserved, allowing a precise definition of the site of the original integration event. tRNA and tmRNA genes are preferred targets of temperate phages and other integrative elements [241-244] (reviewed in [245, 246]), which disrupt the RNA genes by their integration. It has been shown that sRNA genes are also favored targets for integrative elements [247]. In *E. coli*, a bacteriophage P2 *attB* site was found at the 3' end of the *ryeE* gene [32], encoding an Hfq-binding sRNA (recently renamed as CyaR) involved in regulation of multiple targets [69, 73, 77]. In addition, the DNA segment corresponding to the last 23 base pairs of the *ryeB* gene [29, 31, 32] have been reported as the site for multiple integration events (chapter 4). Excision of the integrative element often entails capture of some neighboring host DNA, resulting in an integrative element that carries a copy of the target gene [248]. As preferred target of integration sites, sRNAs are often part of the captured host DNA upon their excision [88, 238]. Therefore, it is not surprising that several sRNA genes have been found in association with integrative elements (chapter 4) [88, 237, 238, 247].

Because they act as insertion hotspots, they constitute horizontally transferred genetic material and also participate in the regulatory cross-talk between exogenous DNA and host core genome, sRNAs can be regarded as key players in bacterial genome evolution.

Objectives

"An unexciting truth may be eclipsed by a thrilling lie."

Aldous Huxley

This thesis work was initiated in 2005, when the sRNA field was blooming. Studies from different laboratories had led to the discovery and characterization of many sRNAs in *E. coli* and *Salmonella*. Nevertheless, most of the targets of sRNA-mediated regulation remained largely unknown at that time. A variety of strategies were being applied to the study of sRNAs, but classical genetic was not represented among them. Since it was already established that Hfq mediates the regulatory activity of the majority of sRNAs, it was decided that, as a starting point, the genetic approach would be used to identify the target genes, taking advantage of this Hfq dependence.

The following objectives were defined:

1. To determine putative targets of sRNA-mediated regulation through genetic studies.

2. To study main features of sRNA structure and function using the regulation of some of the targets identified in (1) as model systems.

3. To explore the possible involvement of sRNAs in biological processes other than post-transcriptional regulation.

Chapter I

Searches for sRNA-regulated genes

During the last decade, many laboratories have developed different computational and experimental strategies to discover, describe an characterize small non-coding RNAs in *E. coli* and *Salmonella* [29-34, 249-259]. Despite the fact that the number of existing sRNAs is considerable and it is still growing, most of their targets were unknown when this thesis work was started. Since then, a variety of approaches have been used to pinpoint sRNAs target candidates, several of which are still being analysed and characterized [250, 256, 260-264].

Because Hfq is known to mediate regulation by *trans*-acting sRNAs [120-122], we decided to undertake a double (genetic and transcriptomic) strategy to search for Hfq-regulated genes, expecting that such a search would identify sRNA-regulated loci. With this purpose, we designed a genetic search using radomly generated translational *lacZ* fusions across the *Salmonella* genome. These fusions were generated in a *hfq* conditional mutant, and permitted the detection of loci in which *lacZ* expression was dependent on Hfq (thus being candidates to be regulated by sRNAs). The screen provided a list of sRNA-regulated genes, some of which were further investigated during the preparation of this thesis (chapters 3 and 4) [4, 52, 118], and led us to discover an unexpected relationship between Hfq and the σ^E stress response (chapter 2).

As an alternative approach, we performed transcriptomic analysis, comparing the global expression profile of an *hfq* mutant strain with that of wild type *S.* Typhimurium. The microarrays experiment provided a list of Hfq-regulated genes expected to include targets of sRNAs regulation and also the sRNAs themselves.

Although genetic screen is briefly explained in chapter 2, both strategies will be described in more detail in this chapter.

I.1. Genetic screening

I.1.1. MudK

MudII1734 (Kan^R, lac) [gene fusion] (MudK) is a phage Mu derivative [265] which harbors deletions of *mudA* and *mudB* genes necessary for transposition and replication. This phage is therefore defective for these functions and must be complemented to transpose [266]. MudK also harbors a Kan^R cassette and a *lacZ* gene (at the "left" end of the phage molecule) without translation initiation site. Thus, transposition of MudK to a protein-coding region generates a translational fusion with the target gene [267]. Because the element is defective, transposition functions must be provided either by a plasmid (in *trans*) [266] or, preferently, from a *cis*-acting transposase gene [267]. Complementation in *cis* can be provided, for instance, by a transposition-proficient Mud prophage (MudI) located near the transposition-defective MudK such that the Mu transposase genes (*A* and *B*) of MudI are close to the defective MudK element (strain called TT10381, table CI.1) [267].

Phage P22 [268] (reviewed in [269, 270]) can package the entire MudK prophage. Fragments carrying the MudK insertion may include the proximal portion of the nearby MudI prophage (and thus the transposase genes) but not the complete MudI element. When this fragment is transduced to a recipient cell, expression of the transposase/replication genes is activated by zygotic induction and transposase acts on the ends of MudK, causing its transposition to the recipient chromosome. The rest of the fragment (including the transposase genes) is eventually degraded or lost by segregation, leaving a single stable insertion free of transposase (figure CI.1) [267].

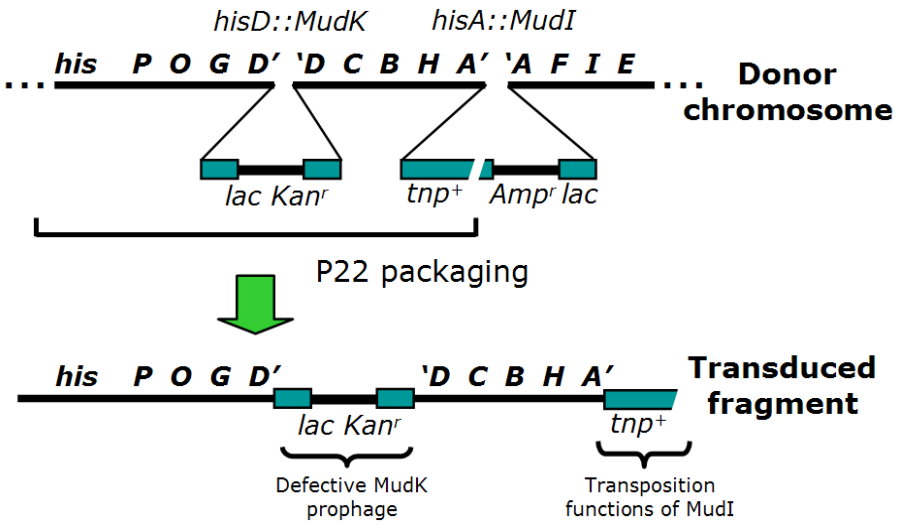


Figure CI.1. Schemathic representation of MudK and MudI region in donor chromosome and transduced fragment in transitory cis complementation events that lead to random MudK insertions in the chromosome (modified from [267]).

I.1.2. Construction of an *hfq* conditional mutant

Hfq is encoded in *amiB-mutL-miaA-hfq-hflX-hflK-hflC* superoperon [271, 272]. The lambda red recombination method [273, 274] was used to replace region between the start (ATG) and stop (TAA) codons of the *hfq* gene with the coding region of the chloramphenicol acetyltransferase (*cat*) cassette, which confers resistance to chloramphenicol. This swap was designed to avoid polarity effects on downstream genes. In the resulting strain, the *cat* cassette is expressed from the native signals that govern *hfq* expression [275, 276]. Because the Hfq protein is abundantly synthesized under most growth conditions, problems related to insufficient ammount of chloramphenicol acetyltransferase were not expected in *Δhfq::cat* strain (MA7791, table CI.1).

To construct a strain carrying an inducible *hfq* gene, the structural portion of the arabinose operon (*araBAD* genes) was replaced with a fragment harboring *hfq* and *aadA* (spectinomycin resistance) genes, using lambda red mediated recombineering. The exchange was designed to have the *hfq* coding sequence starting at the position normally occupied by *araB* (with the downstream *aadA* gene being expressed from autonomous signals). Moving the *ara*::*hfq* fusion into *hfq* deletion strain MA7791 by P22

transduction yielded strain MA7872 (table CI.1), in which the unique copy of the *hfq* gene is under the control of the *araBAD* promoter (P_{BAD}-*hfq*). Thus, Hfq expression in MA7872 strain depends on the presence or absence of arabinose [277-279].

Table CI.1. Bacterial strains

Strain	Genotype
TT10381	*hisD1284*::MudK *hisA9944*::MudI [267]
MA3409	wild-type (Gifsy-1[-]) [280]
MA7791[a]	Δ*hfq67*::*cat* (CmR)
MA7872[a]	Δ*hfq67*::*cat* Δ[*araBAD*]*68*::[*hfq aadA*] (PBAD-*hfq*)

a. Both derived from MA3409

I.1.3. Genetic screen

Strain TT10381 (donated by Kelly Hughes, University of Utah, Salt Lake City) was used to prepare a P22 phage lysate. This lysate includes phages containing the whole MudK element and the left end of MudI, including the transposase/replication genes. The TT10381 lysate was used to transduce strain MA7892 (Hfq conditional mutant) selecting kanamycin resistance (Kan^R). Transductants receiving the MudK element and the transposase gene express the latter transiently, complementing the defective MudK functions and therefore allowing random integration into the recipient chromosome. Kan^R transductants (harboring MudK insertions) were selected either on E medium plates containing glucose and kanamycin or on LB plates with kanamycin. Kan^R colonies were then replica-plated to two sets of LB plates supplemented with X-gal and containing either glucose or arabinose. In some experiments, replica plating was performed on MacConkey plates supplemented with lactose with or without arabinose. The presence or absence of arabinose will determine whether the *hfq* gene will be expressed or not, respectively. Therefore, MudK insertions which exhibit different colour in the presence or absence of arabinose can be expected to lie in putative Hfq-regulated genes. Although indirect effects can be expected, it can be also hoped that some such genes will be regulated by sRNAs.

Colonies showing different colour depending on presence or absence of arabinose were picked and purified by repeated streaking on selective medium. These isolates were used to prepare P22 lysates, which were in turn used to transduce two strains in parallel: MA3409 (wild-type) and MA7791 (*Δhfq*), selecting Kan^R on Lac indicator plates. Transductants showing a colour difference between the Hfq+ and Hfq– isogenic strains were purified and characterized by DNA sequencing and β-galactosidase measurements. Insertions originated from about 10^4 independent transposition events were analysed in this way. Results are discussed in chapter 2.

I.2. Transcriptomic analysis

A complementary approach to perform a genome-wide search of Hfq-regulated genes was RNA expression profile. Using the "Salgenomics" microarrays (see below), the global RNA content of strain MA7791 (*Δhfq*) was compared to that of MA3409 (hfq^+). The experiment was based on the rationale that higher RNA content in a *Δhfq* background would indicate that Hfq represses gene expression in the wild type. In turn, lower RNA content in a *Δhfq* background would indicate that Hfq activates gene expression in the wild type.

I.2.1. Procedures

I.2.1.a. The oligonucleotide-based microarray "Salgenomics"

The microarray 'Salgenomics' was designed for *S. enterica* serovar Typhimurium strain SL1344. Twenty contigs covering >99.5 % of the SL1344 genome sequence were available on November 2004 at the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella). These contigs were used as raw data for the 'Salgenomics' array design. 5,116 open reading frames (ORFs) were predicted using the Glimmer 2.13 program [281]. Of these ORFs, 4,369 were orthologs to STM genes previously annotated in the genome sequence of serovar Typhimurium strain LT2 [13]. Because 4,600 STM chromosomal genes are annotated in LT2, the analysis

predicts a minimum of 94.97% of the LT2 genome content conserved in the SL1344 strain. The set of 4,369 ORFs of SL1344 orthologs to LT2 genes was maintained with the STM nomenclature in our array, to avoid discrepancies with official nomenclature to be assigned by the Welcome Trust Sanger Institute. The SL1344 genes encoding ribosomal RNA were predicted based on those annotated in the LT2 genome sequence and using the algorithm Smith-Waterman implemented in the informatics package FASTAv2.0 (SSEARCH program) [282] Prediction of transfer RNAs (tRNA) was made with the tRNA-SCANSE program [283]. For miscellaneous regulatory and small RNAs (sRNAs), the algorithm Smith-Waterman was used on the LT2 genome database [13]. A total of 21 rRNAs, 86 tRNAS, and 47 sRNA were predicted in the SL1344 genome. sRNAs with no STM identifier assigned in LT2 (5 of the 47 predicted), as well as ORFs predicted in SL1344 but non-matching in the LT2 genome sequence, were assigned identifiers consecutive to CNB1344-0001. These identifiers were assigned based exclusively on the relative positions of the genes in the respective contigs, from 1 to 20, as listed on Nov. 2004 in the Welcome Trust Sanger Institute web page. 70-mer oligo-nucleotides were designed for each of the protein- and RNA-coding genes by using the "OliGO" system (http://www.bioalma.com). Their specificity was examined *in silico* by BLAST analysis and free energies of the oligonucleotide against the second-best match in the SL1344 genome. Each oligonucleotide was spotted in duplicate but in different coordinates (sub-grids) of the array slide. A total of 16 'control' 70-mer oligo-nucleotides designed against non-*Salmonella* bacterial genes 36 and eukaryotic genes were also included and spotted in several sub-grids. Full information on the microarray 'Salgenomics', including spot coordinates and oligo-nucleotide sequences, are deposited in the Miame database (http://www.ebi.ac.uk/miamexpress) with the accession number A-MEXP-846.

I.2.1.b. RNA extractions

To prepare cells for RNA extraction, 25 ml of fresh LB were inoculated with a 1:100 dilution from an overnight bacterial culture and grown in a 250-ml flask incubated with shaking at 250 rpm in a New Brunswick Innova 3100

water bath at 37°C. Three biological replicates were performed for each strain, and cells were collected at an optical density at 600 nm (OD_{600}) 0.9 (see growth curves in figure C1.2). As a somewhat stressful condition, transition from mid-log to stationary phase could trigger expression of many sRNAs. For this reason we decided to collect cells in late exponential growth but before entry into stationary phase, in which Hfq production is not maximal [276] (figure C1.2).

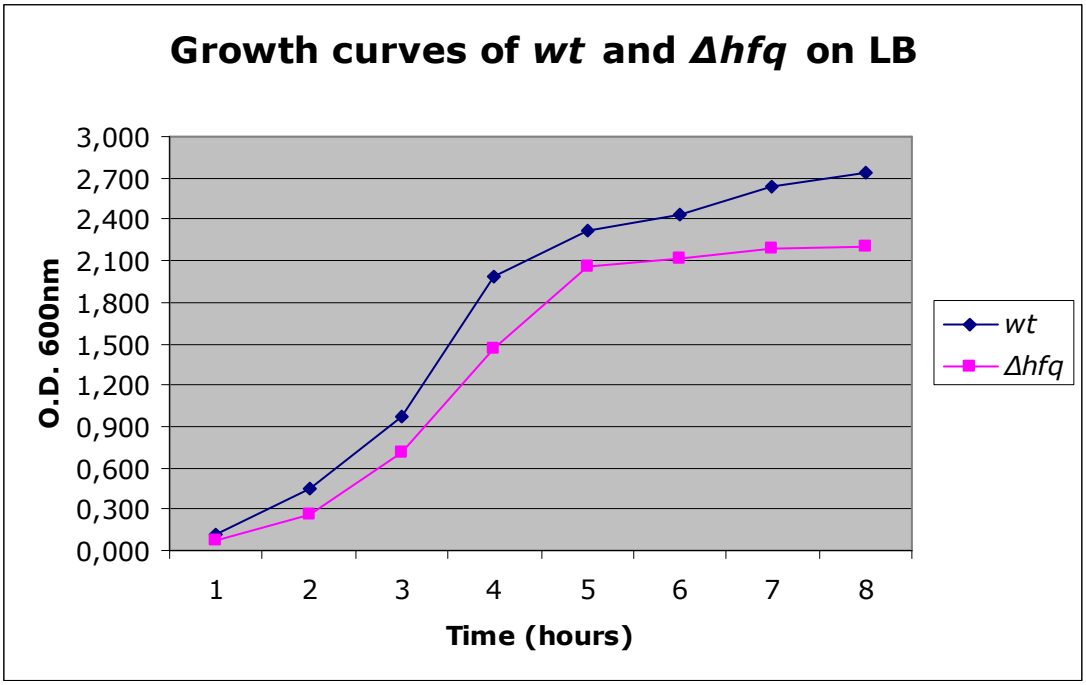


Figure C1.2. Growth curves of MA3409 (*wt*) and MA7791 (*Δhfq*) on LB broth.

RNA extractions were performed as described in [284], with subsequent TURBO™ Dnase (catalog nº. AM2238, Ambion, Inc.) treatment following manufacturer's instructions. RNA quality was assessed on an Agilent 2100 Bioanalyzer.

I.2.1.c. RT and labeling

RNA retrotranscription was performed with Super-Script III reverse transcriptase (catalog no. 18080-044; Invitrogen) according to the instructions of the manufacturer. cDNA was purified with the QIAquick PCR purification kit (catalog no. 28106; Qiagen). Reference gDNA was purified from bacteria grown in 6 ml of LB broth to stationary phase. Upon

centrifugation (10,000 g for 10 min, 4°C), the bacterial pellet was processed using the Qiagen gDNA kit (catalog nº. 19060/10243; Qiagen). DNA was finally suspended in 200 µl of deionized water, resulting in a total amount in the range of 60 to 90 µg. This DNA was further fragmented by sonication before its use in the hybridization assays. Labeling of cDNA and gDNA with Alexa Fluor 647 and Alexa Fluor 555 fluorescence molecules, respectively, was conducted as described previously [285] using exo-Klenow enzyme and the BioPrime Plus array CGH indirect genomic labeling system (catalog no. 18096-011; Invitrogen). Labeled cDNA and gDNA were further purified, and the amount of incorporated labeling was estimated in a NanoDrop ND-1000 UV–visible-light spectrophotometer.

I.2.1.d. Hybridization conditions

Slides were pre-hybridized during 1 h at 42ºC with a solution containing 6X SSC, 0.5% SDS, and 1% bovine serum albumin (BSA). cDNA and gDNA were mixed in a 3:1 ratio (500 and 166 pmoles, respectively). This mixture was reduced to 10 µL final volume, and 70-100 µL of the following solution were added: 50% formamide; 3X SSC, 1% SDS, 5X Denhart's, 5% dextran-sulphate. The solution was then incubated at 95ºC for 5 min. This mixture was added to the slide and the hybridization was continued overnight at 42ºC. After this time, the following washes were made, always in shaking conditions (230 rpm): 3 washes of 5 min each at 42ºC with 0.5X SSPE (20X SSPE: 175.3 g/L NaCl, 27.6 g/L NaH2PO4, 7.4 g/L Na2EDTA pH 7,4), 0.1% Tween20; 3 washes of 5 min each at 42ºC with 0.5X SSPE; and two final washes of 5 min each with 0.1X SSPE at 37ºC. The slides were rinsed with distilled water before drying in the centrifuge during 1 min at 600 x *g*.

I.2.1.e Data acquisition, normalization and statistical analysis

Images from Cy3 and Cy5 channels were equilibrated and captured with a GenePix 4000B (Axon) and converted into numerical data using GenPix Pro 5.1 software (Axon Instruments). Subsequent data analyses were carried-out in R (http://r.project.org) using *limma* [286] and *affy* [287] packages of the Bioconductor project (http://bioconductor.org). Spot intensities were

background-corrected using the *normexp* option in *limma* with an offset of 50. Background-corrected intensities were log transformed (base 2). To compensate systematic deviations of spot intensities due to different labelling and/or detection efficiencies in the replicated microarrays, background-corrected log intensities were normalized using the 'normalize.quantiles' function of *affy* package. This normalization method adjusts the quantiles of each set of intensities, so their distributions become similar [288]. The process was carried-out separately for Cy3 (coming from cDNA) and Cy5 (coming from genomic DNA) intensities. The normalized values were named 'log-RNA-signal' and 'log-Genomic signal', respectively. Log-Genomic-signals served as good estimation of the amount of DNA present in each spot in the microarray, and they were used to correct the differences in the log-RNA signal due to the differences in the amount of DNA:

Final logRNAsignal (logRNAsignalF) = logRNAsignal – logGenomicSignal = log (RNAsignal/GenomicSignal)

The logRNAsignalF was quantile-normalized again to minimize any remaining intensity bias between replicates. Using the logRNAsignalF, a linear model was fitted for *each* gene using the *limma* package and differential expression between RNA sample X and RNA sample Y were estimated. Differentially expressed genes were identified using the moderated *t*-statistic in *limma* and, correcting for multiple testing and by calculating the False Discovery Rate (FDR) as described [289]. Changes in expression for a certain gene were considered significant when the log2 of the ratio between sample X and Y (M value) was ≤ -2 or ≥ 2 and the *p* value lower than 0.05. The analysis treated each spot of the array as a different gene, performing such comparison between spots of different biological samples (X and Y) but in identical coordinates. Only when the two duplicates of a certain gene positioned in different coordinates in the microarray slide had an M value ≤ -2 or ≥ 2, the changes in expression for such gene were considered significant. FIESTA viewer (http://bioinfogp.cnb.csic.es/tools/FIESTA) was used to graphically visualize the result of applying the different statistical filters to the values. Given the type of hybridization used, cDNA versus genomic DNA, data processing

allowed comparisons among multiple strains and/or growth conditions as described previously [285].

I.2.2. Results and discussion

Overall, RNA levels of 29 genes were >2-fold higher and RNA levels of 55 genes were >2-fold lower in Hfq$^-$ mutant. Higher RNA levels in an Hfq$^-$ background indicated that Hfq represses gene expression in the wild type (Table 2) , whereas lower RNA ammounts in an Hfq$^-$ background indicated that Hfq activates gene expression in the wild type (Table CI.3). The relevant transcriptomic data can be summarized as follows.

- **Virulence-related genes**. A number of virulence related genes showed decreased expression in an *hfq* background. The list includes genes encoded on pathogenicity islands SPI-1, SPI-3 and SPI-4 (Table CI.3). Involvement of Hfq in virulence processes in many bacteria is well known (reviewed in [106]). Our data underlined the importance of Hfq in *Salmonella* virulence (it has been described that an *hfq* mutant is more than 100-fold atenuated in mice [65]), as has been subsequently ratified by others [290].

- **Genes encoding membrane-associated proteins**. Genes whose products are part of the bacterial envelope showed differential Hfq-dependent expression, confirming the close relationship between cell membrane and Hfq-mediated regulation by sRNAs (reviewed in [72, 81-83]).

- **Flagellar system genes**. Genes enconding proteins of the flagellar apparatus are also activated by Hfq. These results are agree with motility impairement detected in *Salmonella hfq* mutants described by latter studies [65, 290].

- **Conjugal-transfer genes**. The transfer operon (*tra*) of the virulence plasmid (pSLT) showed increased expression in an

hfq host, confirming a previous report on other for the F sex factor in *E. coli* [291].

- **Others**. Genes related to metabolism, regulation of gene expression and DNA modification also showed Hfq-dependent regulation, thereby emphasizing the key role of the Hfq RNA chaperon in bacterial cell physiology (reviewed in [106, 292, 293]).

Despite the involvement of Hfq in sRNA-mediated regulation, some genes are regulated by Hfq in an indirect fashion. For instance, because Hfq is required for efficient translation of *rpoS* mRNA [112-116], expression of RpoS-regulated genes can be affected by Hfq. Thus microarrays data include as Hfq-regulated genes some that are not directly regulated by Hfq nor sRNAs but are RpoS-dependent. Genes like *acs* [294] and *narY* [295] are examples of this indirect regulation (Table CI.3).

Indirect regulation by Hfq is also caused by its effect on the σ^E regulon. Lack of Hfq causes constitutive activation of σ^E-dependent envelope stress response (extensively described in chapter 2). Hence, genes whose transcription is controlled by σ^E are expected to show increased expression in an *hfq* background. Is known that *rpoE* gene, encoding σ^E, undergoes autogenous positive regulation (chapter 2) [178, 181, 290]. Therefore, is not surprising that *rpoE* gene appears upregulated in the *hfq* mutant (Table CI.2).

Table CI.2. Genes downregulated by Hfq

Gene	Protein function	Fold change[a]
ackA	acetate/propionate kinase	2.37
dpiB (citA)	citrate-proton symporter	2.09
dppA	dipeptide transport protein	2.11
lamB	maltoporin precursor	3.11
malM	periplasmic protein precursor	2.01
ptsG	glucose-specific IIBC component	2.19
pyrB	aspartate carbamoyltransferase catalytic subunit	2.03
rpoE	RNA polymerase sigma-E factor	2.39

STM1036	probable minor tail protein (phage Gifsy-2)	2.22
STM1254	putative outer membrane lipoprotein	6.22
STM1538	putative hydrogenase-1 large subunit	2.15
STM2426	putative cytoplasmic protein	2.09
traW	pilus assembly protein	2.04
treB	trehalose (maltose)-specific PTS enzyme: IIB/IIC component	4.82
treC	trehalose-6-phosphate hydrolase	4.34
treR	trehalose repressor	2.66
xni	exonuclease IX	2.23
chiP (ybfM)	chitoporin	3.44
chiQ (ybfN)	putative lipoprotein	2.00
rsmF (yebU)	rRNA methyltransferase	2.10
norW (ygbD)	nitric oxide reductase	2.13
yihW	putative glycerol-3-phosphate regulon repressor	2.65

Putative ORF	**Locus**	**Fold change[a]**
S1507A_ORF	STM1254 (comp)/STM1255	5.12

IGR	**Locus**	**Fold change[a]**
S5360B_IGR	complement, *glgS* (comp)*/STM3198* IGR	2.02
S5744B_IGR	complement, *pepB* (comp)*/ yfhJ* (comp) IGR	2.16
S5833B_IGR	*glnS/ybfM* IGR	2.12
S5960B_IGR	complement, *treR* (comp)*/mgtA* IGR	2.12
S5982A_IGR	complement, *STM4320/pheR*(comp) IGR	2.50
S6101A_IGR	complement livJ (comp)/rpoH (comp) IGR	2.43

a *wild-type/hfq* mutant expression ratio

I.2.2.a. Genes that are downregulated by Hfq.

A variety of cellular functions are affected by Hfq-mediated repression. For example, the list on table CI.2 includes a gene enconding a rRNA methyltransferase, *rsmF* (formerly *yebU*) [296], the *rpoE* gene (chapter 2) [290, 297], genes with virulence-related roles and, specially, genes enconding membrane proteins.

Virulence-related functions

Resistance to stress has shown to be an important feature in bacterial pathogenesis (reviewed in [298]). Hfq participates in virulence functions

both directly, controlling expression of many virulence factors (see below) [65, 290] (reviewed in [106]) and indirectly, affecting the expression of genes involved in resistance to multiple stresses (chapter 2) [65, 168, 290]. Examples of stress-related functions under Hfq control include the acetate/propionate kinase AckA, which has been shown to be involved in invasion, affecting expression of SPI-1 [234, 299]. Another example of an Hfq-regulated stress response gene is *norW* (formerly *ygbD*) gene, which encodes a nitric oxide reductase involved in NO detoxification [300].

Hfq-regulation of pSLT-encoded *tra* operon could affect virulence in more than one way. The *tra* operon encodes several proteins involved in conjugal transfer of virulence plasmid pSLT, which also harbors genes that participate in pathogenesis [21-23]. Microarray data indicate that Hfq regulates the pSLT *tra* operon, as previously described for F plasmid in E.coli [291]. In addition to conjugal transfer, the *tra* operon of F plasmid also play a role in resistance to bile salts in *E. coli* [301]. Thus, Hfq might contribute to virulence through *tra* operon regulation both by promoting pSLT conjugation and by increasing resistance to bile salts.

Biofilms largely contribute to stress resistance and virulence in many organisms (reviewed in [302, 303]). Transcriptomic analysis showed that *yihW* is regulated by Hfq. The YihW protein is involved in assembly and translocation of O-antigen capsule, which is specifically required for biofilm formation of *Salmonella* on cholesterol gallstones [304]. In *E. coli*, transcriptional activator of curli fimbriae formation, CsgD (AgfD), also acts on the *yihVW* operon. CsgD transcription is activated by OmpR, which is a target of OmrA/OmrB sRNAs [71]. In addition, CsgD has been recently described as direct target of OmrA/OmrB as well [60]. Given that OmrA/OmrB sRNAs require Hfq for their action, Hfq-mediated regulation of *yihW* might result from increased transcription, caused by relieving OmrA/OmrB-dependent repression of CsgD.

Regulation of stress resistance by Hfq, together with Hfq-mediated activation of essential virulence factors (see below) [290], underline the key role of Hfq protein in regulation of virulence processes (reviewed in [106]).

Membrane proteins

Genes encoding membrane-associated proteins are the major class among Hfq-repressed genes. Some such genes have been shown to be inhibited by sRNAs with the participation of Hfq. These include *dppA* (encoding a dipeptide transport system) which is repressed by the sRNA GcvB [61, 305], *ptsG* (major glucose transporter), regulated by SgrS sRNA [28, 306-310], *lamB-malM* (the product of processing a larger *malK-lamB-malM* transcript),encoding a maltoporin precursor and a periplasmic protein, respectively, which is repressed by MicA [4], *chiPQ* (enconding a chitoporin and a putative lipoprotein, respectively) and *dpiB* (enconding a citrate-proton symporter) transcripts, both repressed by ChiX sRNA [52, 76, 78, 311].

Thus microarray data confirm the extensive role of sRNAs on the homeostatic control of bacterial membrane elements (reviewed in [72, 81-83]).

Intergenic region data

Inclusion of intergenic regions in the microarray allows detection of potential unknown transcripts whose expression could be affected by Hfq. *A priori*, those could include both mRNAs and sRNAs.

Unknown function genes

The list of Hfq-activated genes also include some *Sallmonella*-specific genes of unknown function. Among them, *STM1254*, which encodes a putative outer membrane lipoprotein, shows strong Hfq dependence. Interestingly, the region complementary to IGR between *STM1254* and *STM1255* shows Hfq-dependence to a comparable extent. In addition, this region contains an ORF enconding a putative small protein (45 aminoacids) present in several other serovars including *Salmonella* Typhi. Different mechanisms of Hfq-mediated regulation are possible in this scenario.

Table CI.3. Genes upregulated by Hfq

Gene	Protein function	Fold change[b]
acs	acetyl-coenzyme A synthetase	2.03
aspA	aspartate ammonia-lyase	2.14
astA	arginine succinyltransferase	2.59
creC	sensor kinase	2.33
dapB	dihydrodipicolinate reductase	2.70
fliK	flagellar hook-length control protein	2.03
fljB	flagellar filament structural protein	2.28
gltA	citrate synthase	2.31
hfq	RNA-binding protein Hfq	2.56
hilA	invasion protein transcriptional activator (SPI-1)	2.26
hilC	invasion regulatory protein (SPI-1)	2.41
hilD	invasion protein regulatory protein (SPI-1)	2.31
invA	needle complex export protein (SPI-1)	2.69
invB	secretion chaperone (SPI-1)	2.32
invC	type III secretion system ATPase (SPI-1)	3.82
invF	invasion regulatory protein (SPI-1)	2.77
invG	outer membrane secretin precursor (SPI-1)	2.14
invI	needle complex assembly protein (SPI-1)	2.71
misL	putative autotransporter (SPI-3)	2.38
narY	nitrate reductase 2 beta subunit	2.60
prgH	needle complex inner membrane protein (SPI-1)	2.99
prgI	needle complex major subunit (SPI-1)	3.98
prgJ	needle complex minor subunit (SPI-1)	3.60
prgK	needle complex inner membrane lipoprotein (SPI-1)	2.28
sdhA	succinate dehydrogenase catalytic subunit	2.56
sicA	secretion chaperone (SPI-1)	2.77
sipA	secreted effector protein (SPI-1)	2.10
sipB	translocation machinery component (SPI-1)	3.27
sipC	translocation machinery component (SPI-1)	2.65
sipD	translocation machinery component (SPI-1)	2.97
stbB	putative fimbrial chaperone (pSLT)	2.20
STM0257	putative drug efflux protein	2.17
STM0356	putative inner membrane protein	2.83
STM1549	putative translation initiation inhibitor	2.20
STM3631	putative xanthine permease	2.08
STM4258	putative methyl-accepting chemotaxis protein (SPI-4)	2.95
STM4259	putative ABC exporter outer membrane component (SPI-4)	2.38
STM4260	predicted cation efflux pump (SPI-4)	2.13
wraB	TrpR binding protein WraB	2.13
ybaZ	alkyltransferase involved in NER	2.06
bsmA (yjfO)	putative lipoprotein related to biofilms, stress and motility	2.40

Putative ORF	Locus	Fold change[b]

S1107A_ORF	complement, *STM1629/STM1630*	2.58
S3621A_ORF	*iraP (yaiB)/psiF*	2.55
S4320A_ORF	*ecnR* (complement)/*sugE*	2.02

IGR	**Locus**	**Fold change[b]**
S5496A_IGR	*purR* (comp)/*sodB* (comp)	2.09
S5514A_IGR	*osmE/celA* IGR	2.53
S5807A_IGR	complement, *sprB* (comp)/*hilC* (comp) IGR (SPI-1)	2.82
S5809A_IGR	complement, *prgH* (comp)/*hilD* (comp) IGR (SPI-1)	2.01
S5810B_IGR	complement, *hilD* (comp)/*hilA* (comp) IGR (SPI-1)	2.84
S5855A_IGR	complement, *folD* (compl)/*fimA* IGR	3.02
S5879B_IGR	*STM0327/STM0328* IGR	2.45
S5934B_IGR	complement, *STM4597* (comp)/*arcA* (comp) IGR	2.03
S5937A_IGR	*prfC/osmY* IGR	2.17
S5997A_IGR	complement, *ssb-STM4257/STM4258* IGR (SPI-4)	3.52
S6065B_IGR	marT/STM3760 (SPI-3)	3.83

b *hfq* mutant/*wild-type* expression ratio

I.2.2.b. Genes activated by Hfq.

Some genes whose products are involved in metabolism show positive regulation by Hfq. For instance, *acs*, enconding acetyl-coenzyme A synthetase, was previously described as RpoS-dependent. Given that Hfq is necessary for fully efficient translation of *rpoS* [112-116], is not surprising that *acs* transcript is less abundant in an *hfq* background. The same occurs with *narY* (which encodes nitrate reductase 2 beta subunit), which belongs to the RpoS-dependent *narZYWV* operon [290, 295]. Genes encoding aspartate ammonia-lyase (*aspA*) and Trp binding protein WraB (*wraB*) have been also described by other authors as Hfq-activated [290].

Virulence-related functions

Beside genes whose products are involved in metabolism and environmental awareness, the list of Hfq-activated genes includes genes directly or indirectly related to virulence, as those involved in flagellar synthesis, DNA repair, stress resistance, biofilm formation and overall genes belonging to *Salmonella* pathogenicity islands 1, 3 and 4. The list also includes the *hfq* gene itself, in agreement with the previously described translational

autogenous control of Hfq in *E. coli* [123] and with a recent transcriptomic study in *Salmonella* [290].

An example of Hfq-dependent DNA repair gene is *ybaZ*, which encodes an alkyltransferase involved in NER [312]. Hfq-mediated activation of *ybaZ* has been confirmed by others [290], and could represent another indirect mechanism for Hfq-mediated regulation of *Salmonella* virulence. Indirect evidence for the participation of NER in *Salmonella* virulence has been previously described [313, 314].

Our study shows the flagellar genes *fljB* (encoding a flagellar filament structural protein) and *fliK* (encoding flagellar hook-length control protein) as Hfq-activated, in agreement with a recent study [290]. Motility and flagellar synthesis are also essential for bacterial pathogenesis (reviewed in [315]), and implication of Hfq in this processes has been independently ratified [65].

BsmA is a protein involved in biofilm formation, acid and peroxide stress resistance and motility [316]. The *bsmA* gene is activated by Hfq in our study, an observation confirmed by others [290].

The most relevant contribution of Hfq to virulence involves pathogenicity islands. In agreement with recent studies [65, 290], our microarray analysis showed Hfq-mediated activation of genes in SPI-1, SPI-3 and SPI-4. SPI-1 and SPI-4 encode functions necessary for adhesion and invasion of intestinal epithelial cells [317] (reviewed in [318]). Co-ordinated expression of SPI-1 and SPI-4 is necessary for optimal invasion [227, 229, 319-321]. SPI-3 encodes genes whose products participate in macrophage survival [322].

Intergenic region data

Microarray data of Hfq-activated IGRs could include both mRNAs regulated positively by Hfq (through sRNAs or not) and/or sRNAs themselves. Given that Hfq is necessary for stability of sRNAs [33, 93], it can be expected that

RNA levels of sRNAs-encoding IGRs will be higher in Hfq^+ background, showing an "Hfq-activated" pattern. On the other hand, IGRs belonging to Hfq-activated operons will concomitantly show the corresponding regulation; that is probably the case for S5807A_IGR and S5809A_IGR, corresponding to *sprB/hilC* and *prgH/hilD* IGRs. Since the entire SPI-1 is under the positive control of Hfq, those IGRs probably just reflect higher ammounts of SPI-1 transcripts in an Hfq^+ background. The same is likely to occurs with S6065B_IGR, located in SPI-3 (*marT/STM3760*).

Interestingly, S5496A_IGR and S5810B_IGR (corresponding to *purR/sodB* and *hilD/hilA*, respectively) have been described as putative sRNAs (respectively called STnc550 and STnc600) by pyrosequencing and co-immunoprecipitation with Hfq [290]. Curiously, a third IGR called S5997A_IGR comprises the region complementary to the STM4257 gene (SPI-4) and also to another putative sRNA called STnc620 [290]. Given that sRNAs genes are hotspots for horizontally adcquired material (chapter 4) [32, 247] and they have been frequently found at the ends of genomic islands [88, 237, 238], is tempting to interprete our data as evidence for the presence of a previously unknown SPI-4-encoded sRNA.

In summary, this unpublished transcriptomic study shows involvement of Hfq in a variety of cellullar functions, including multiple contribution to virulence (activating pathogenicity islands, regulating biofilm formation, motility and stress resistance) and controlling the homeostatic regulation of bacterial envelope.

I.3. Conclusions: initial stage for further studies

The double genetic/transcriptomic strategy revealed the extensive involvement of Hfq and sRNAs in a variety of essential physiological processes. It also helped to clarify *hfq* phenotypes. The overall results from the genetic screen and microarrays analysis prompted us to study several systems in which Hfq and sRNA seemed to play a key regulatory role (chapters 2 and 3).

Chapter II

Loss of Hfq activates the σ^E–dependent envelope stress response in *Salmonella enterica*

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Loss of Hfq activates the σ^E-dependent envelope stress response in *Salmonella enterica*

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Summary

Ubiquitous RNA-binding protein Hfq mediates the regulatory activity of many small RNAs (sRNAs) in bacteria. To identify potential targets for Hfq-mediated regulation in *Salmonella*, we searched for *lacZ* translational fusions whose activity varied in the presence or absence of Hfq. Fusions downregulated by Hfq were more common than fusions showing the opposite response. Surprisingly, in a subset of isolates from the major class, the higher activity in the absence of Hfq was due to transcriptional activation by the alternative sigma factor RpoE (σ^E). Activation of the σ^E regulon normally results from envelope stress conditions that elicit proteolytic cleavage of the anti-σ^E factor RseA. Using an epitope tagged variant of RseA, we found that RseA is cleaved at an increased rate in a strain lacking Hfq. This cleavage was dependent on the DegS protease and could be completely prevented upon expressing the *hfq* gene from an inducible promoter. Thus, loss of Hfq function appears to affect envelope biogenesis in a way that mimics a stress condition and thereby induces the σ^E response constitutively. In a RseA mutant, activation of the σ^E response causes Hfq-dependent downregulation of outer membrane protein (OMP) genes including *lamB*, *ompA*, *ompC* and *ompF*. For *ompA*, downregulation results in part from σ^E-dependent accumulation of MicA (SraD), a small RNA recently shown to downregulate *ompA* transcript levels in stationary phase. We show that the *micA* gene is under σ^E control, and that DegS-mediated σ^E release is required for the accumulation of MicA RNA upon entry into stationary phase. A similar mechanism involving additional, still unidentified, sRNAs, might underlie the growth phase-dependent regulation of other OMP mRNAs.

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Introduction

Regulatory mechanisms involving small non-coding RNAs have received considerable attention in recent years. In the area of prokaryotic gene regulation, the ability of small RNAs (sRNAs) to mediate rapid post-transcriptional adjustments is increasingly regarded as a factor promoting bacterial adaptation to environmental changes. A major class of regulatory sRNAs in *Escherichia coli* are encoded at distant locations from their sites of action and act by base-pairing with partially complementary sequences in the 5′ untranslated regions (5′-UTR) of target mRNAs (Gottesman, 2002; 2004; Wagner *et al*., 2002; Wagner and Vogel, 2003; Storz *et al*., 2004; Storz *et al*., 2005). The duplex structure most often downregulates gene expression by interfering with loading of ribosomes onto the mRNA and/or by causing the mRNA to be more rapidly degraded (Massé *et al*., 2003; Morita *et al*., 2005, 2006; Rasmussen *et al*., 2005; Udekwu *et al*., 2005). An exception to this rule is found in the regulation of *E. coli rpoS* (σ^S) mRNA translation (Repoila *et al*., 2003; Majdalani *et al*., 2005). Binding of either DsrA or RprA sRNAs prevents the *rpoS* mRNA leader sequence from adopting a secondary structure that occludes the ribosome binding site. Thus, in this particular case, the sRNA interaction serves to stimulate translation initiation. Most, if not all, sRNAs that act by the above mechanism do so in complex with the chaperon protein Hfq (Valentin-Hansen *et al*., 2004). Hfq often binds both regulator and target RNAs and is required for the regulatory response. The protein was shown to favour the interaction between the RNA partners (Møller *et al*., 2002; Zhang *et al*., 2002) and to enhance stability of many sRNAs *in vivo* (Moll *et al*., 2003; Zhang *et al*., 2003). Because of the difficulty in distinguishing between these effects, the role of Hfq in sRNA-mediated regulation remains incompletely understood.

A significant proportion of sRNA-controlled genes in *E. coli* encode components of the bacterial envelope. Notably, they include the genes for major outer membrane proteins (OMPs), OmpA, OmpC and OmpF,

regulated, respectively, by MicA (previously SraD) MicC and MicF sRNAs (Mizuno *et al*., 1984; Chen *et al*., 2004; Rasmussen *et al*., 2005; Udekwu *et al*., 2005). All three sRNAs act by base-pairing to the leader regions of cognate target mRNAs, inhibiting translation and promoting mRNA decay. In concert with transcriptional regulation, this mechanism contributes to adjusting the levels of the three proteins to environmental conditions. For instance, MicF mediates OmpF variations in response to high temperature, weak acids and oxidative stress (Delihas and Forst, 2001) while MicA is involved in the growth-phase-dependent regulation of OmpA (Rasmussen *et al*., 2005; Udekwu *et al*., 2005). Co-ordination in the expression of *omp* genes seems critical for proper envelope assembly. Mutations or other conditions that unbalance OMP levels activate the σ^E response, a complex set of changes normally devoted to protecting the envelope from environmental insults (Alba and Gross, 2004; Ruiz and Silhavy, 2005). The response is triggered by the accumulation of unfolded or misfolded OMP precursors in the periplasm (Mecsas *et al*., 1993). This signal is transduced across the inner membrane in the form of a proteolytic cascade that releases alternative sigma factor σ^E from its membrane-associated captor, the RseA protein (De Las Peñas *et al*., 1997; Missiakas *et al*., 1997; Ades *et al*., 1999). The cascade involves two distinct proteases acting sequentially. Inner-membrane anchored DegS protein cleaves RseA in its periplasmic domain, followed by the action of membrane-embedded RseP (YaeL) protease which cleaves RseA at the level of its inner membrane segment (Alba *et al*., 2002; Kanehara *et al*., 2002; 2003; Walsh *et al*., 2003). An additional factor, RseB, increases the specificity and robustness of the response by protecting RseA from gratuitous degradation (Grigorova *et al*., 2004). Once free in the cytoplasm, σ^E associates with RNA polymerase core enzyme and activates transcription of a set of genes that collectively help the bacterium to recover from the stress condition (Rezuchova *et al*., 2003; Skovierova *et al*., 2006).

Knowledge of sRNA-mediated regulation in *Salmonella* species remains limited although the presence of homologues to many *E. coli* sRNA genes in *Salmonella* genomes suggests the existence of conserved mechanisms. To identify genes potentially regulated by sRNA in *Salmonella*, we screened a library of random *lacZ* chromosomal inserts searching for translational fusions whose β-galactosidase activity changed in a strain expressing the *hfq* gene conditionally. Characterization of the isolates obtained revealed that a subset of Hfq-downregulated genes belonged to the σ^E regulon, prompting us to analyse the role of Hfq in the σ^E response, as well as the interplay of Hfq and σ^E in the regulation of major OMPs.

Results

Loss of Hfq function activates σ^E-dependent transcription in Salmonella

A *Salmonella enterica* serovar Typhimurium strain carrying a non-polar *hfq* deletion and a copy of the *hfq* gene under the control of the chromosomal pBAD promoter was constructed as described in *Experimental procedures*. This strain (MA7872) was used as a recipient for transposition of the MudK element, a phage Mu derivative which transposes largely at random and generates *lacZ* gene fusions upon inserting within coding regions (Hughes and Roth, 1988). Colonies resulting from transposition were replica-plated on Lac indicator plates with and without arabinose. Isolates whose colour was different in the presence or absence of arabinose were purified and each MudK insertion was transferred by transduction into a pair of isogenic Hfq^+ and Hfq^- strains. Insertions maintaining a colour difference were characterized by DNA sequencing and β-galactosidase measurements. Forty-five isolates, originating from the screening of about 10^4 independent transposition events, were analysed in this way. Twenty-three insertions showing the most reproducible differences are described here. As shown in Fig. 1, a majority of these fusions is upregulated in the *hfq* mutant background. Intriguingly, most isolates from this class carry *lacZ* inserted in genes encoding known or putative components of the bacterial envelope (Table 1). These include genes for porins (*lamB*, *ybfM*) transporter proteins (*dppF*, *gudT*, *sbmA*, STM1543, *yifK*), enzymes involved in LPS biosynthesis or modification (*eptB*, *yihG*), membrane-associated metabolism (*aphA, dmsA*), conjugal plasmid transfer (*traT*) and unknown fuctions (*ycbK*). In addition, insertions in metabolic genes (*pduD*, *pduO*), as well as in the *rpoE-rseABC* operon (*rseC*) and in the gene for a putative LysR-type transcriptional regulator (*yhjC*) are part of the upregulated group. A handful of fusions show the opposite response and are expressed to a lower level in the *hfq* mutant (Fig. 1). Three out of the four members of this class (*gabP*, *katE* and *sufE*) lie within genes transcribed by stationary-phase sigma factor RpoS (σ^S) (Vijayakumar *et al*., 2004; our unpublished results). Conceivably, the response of these fusions reflects the requirement for Hfq in the activation of *rpoS* mRNA translation by DsrA and RprA sRNAs (Majdalani *et al*., 2005).

In the isolates from the major group, Hfq downregulates *lac* expression. To gain insight into the mechanism(s) involved, we sought mutations relieving negative control. A strain harbouring one of the fusions with the highest activation ratio (*eptB*::*lacZ*; Fig. 1) in an otherwise wild-type background, was subjected to Tn5-T-POP mutagenesis and clones with increased β-galactosidase activity were identified and characterized. Mutants were found to carry the transposon inserted at either of two loci: the *hfq*

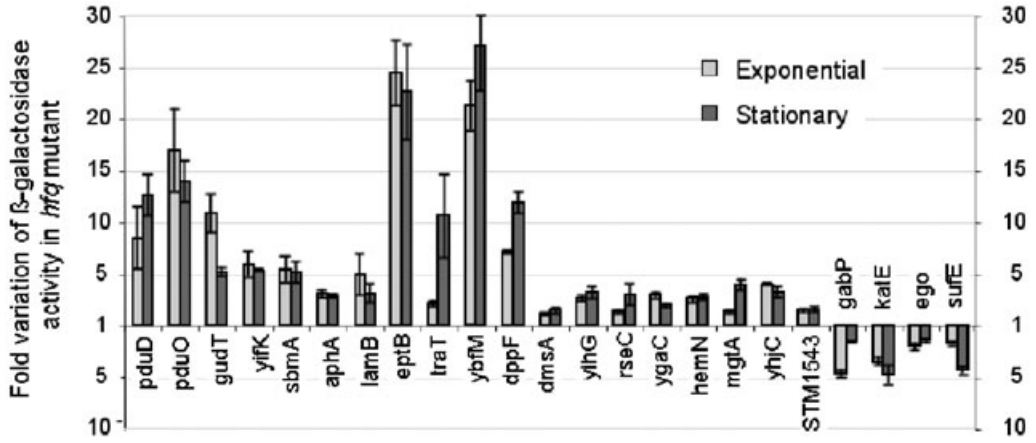

Fig. 1. Response of *lacZ* gene fusions to *hfq* inactivation. Fusions identified as described in the text were transferred to isogenic strains MA3409 (*hfq*$^+$) and MA7791 (Δ*hfq*). Cultures were grown to an OD_{600} = 0.3–0.7 (exponential) or overnight (stationary) and β-galactosidase activity was measured as described by Miller (1992). The histograms represent ratios between β-galactosidase activities in Hfq$^-$ and Hfq$^+$ backgrounds. Negative ratios indicate β-galactosidase activities that were lower in the Hfq$^-$ background.

gene and the gene encoding the anti-σ^E factor RseA. Like in *E. coli*, the *rseA* gene of *Salmonella* is located immediately downstream from the σ^E-encoding *rpoE* gene (McClelland *et al*., 2001). These findings suggested that the *eptB* gene, which encodes an LPS modifying enzyme (Reynolds *et al*., 2005), was under σ^E control and that its upregulation in the *hfq*$^-$ background reflected activation of σ^E-dependent transcription. A deletion of the entire *rseA* coding sequence (obtained by exchange with a spectinomycin resistance (Spc^R) cassette) gave identical results (Fig. 2A). The *rseA*-deleted strain (MA8386) formed red colonies on MacConkey Lac indicator plates. When left at room temperature for several days, these colonies gave rise to white (Lac$^-$) papillae. Characterization of several Lac$^-$ segregants showed that most of them carried *rpoE* point mutations that sharply lower σ^E activity (N. Figueroa-

Table 1. Hfq-regulated genes identified in this study.

Gene[a]	Function	Length (bp)[b]	MudK starts (bp)[c]
pduD	Propanediol dehydratase subunit	672	52
pduO	ATP:cob(I)alamin adenosyltransferase	1008	826
gudT	D-glucarate permease	1356	838
yifK	Putative amino acid transport protein	1383	142
sbmA	Inner membrane transport protein (microcin J25 sensitivity)	1221	1192
aphA	Acid phosphatase	711	2
lamB	Maltoporin	1356	943
eptB	Inner membrane phosphoethanolamine transferase	1689	448
traT	Conjugative transfer surface exclusion protein (pSLT plasmid)	732	526
ybfM	Putative outer membrane porin, OprD family	1404	615
dppF	Dipeptide transport protein	1011	670
dmsA	Anaerobic dimethyl sulphoxide reductase chain A	2427	2068
yihG	Phosphate acyltransferase (phospholipid biosynthesis)	906	34
rseC	σ^E regulator	477	34
ygaC	Conserved hypothetical protein	348	196
hemN	Oxygen-independent coproporphyrinogen III oxidase	1371	64
mgtA	Magnesium ATPase transporter	2706	1168
yhjC	Putative LysR-type transcriptional regulator	897	409
STM1543	Putative sugar transport protein	1284	646
gabP	Gamma-aminobutyrate transport protein	1398	16
katE	Catalase	2250	460
ego	Putative ABC-type sugar aldose transport ATPase	1533	1267
sufE	Cysteine desulphuration protein	414	118

a. MudK insertion sites (see text) were determined by sequencing DNA fragments amplified by inverse PCR of chromosomal DNA (Hartl and Ochman, 1996). Genes were identified by matching the sequence data with the annotated *Salmonella enterica* serovar Typhimurium strain LT2 genome sequence (McClelland *et al*., 2001).
b. Length of coding sequence.
c. Distance between the start of the coding sequence and the MudK element. All insertions generate in-frame fusions to *lacZ*, except in *aphA* and *ybfM (oprD*) genes where LacZ activity presumably results from translation initiating at the insertion site.

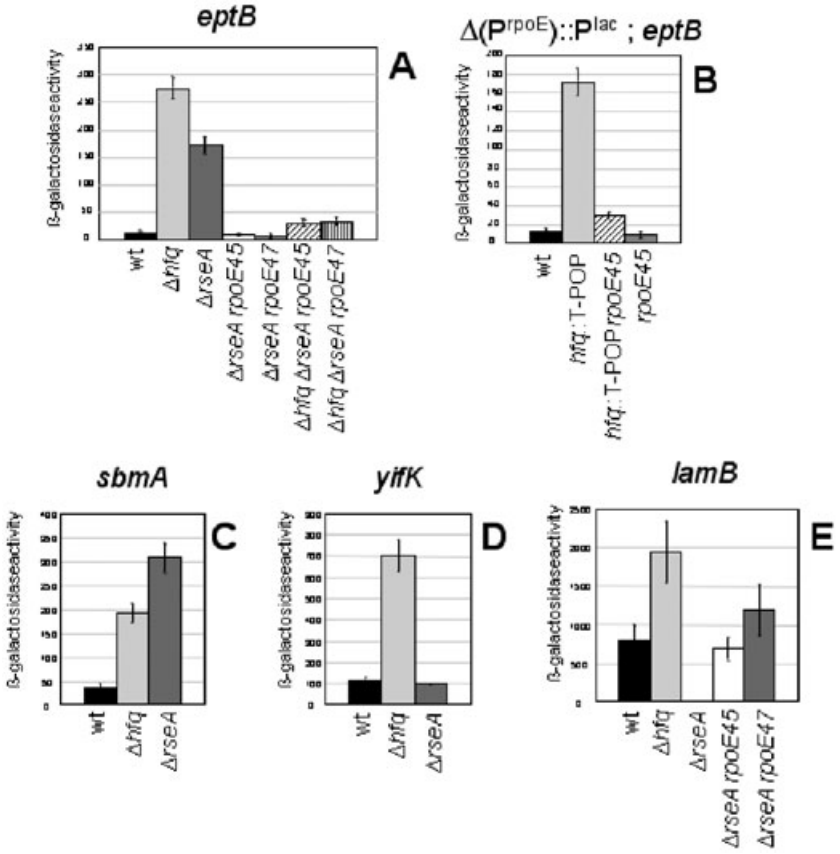

Fig. 2. Effect of Hfq or σ^E alterations on expression of representative *lacZ* gene fusions. β-Galactosidase activity was measured in cultures with OD_{600} between 0.3 and 0.7 as described by Miller (1992). The values shown are the averages of at least two independent determinations.
A. Regulation of the *eptB*::*lacZ* fusion. Strains: MA8028, MA8029, MA8386, MA8471, MA8472, MA8798, MA8799.
B. Regulation of the *eptB*::*lacZ* fusion in the background of a *rpoE*/*lac* promoter swap ($\Delta P^{rpoE}::P^{lac}$). Strains: MA8818, MA8806, MA8816, MA8814.
C. Regulation of the *sbmA*::*lacZ* fusion. Strains: MA8022, MA8023, MA8385.
D. Regulation of the *yifK*::*lacZ* fusion. Strains: MA8020, MA8021, MA8433.
E. Regulation of the *lamB*::*lacZ* fusion. Strains: MA8026, MA8027, MA8546, MA8744, MA8745. The full genotypes of strains used are in Table 2.

Bossi and L. Bossi, unpubl. data). Two such mutants, harbouring alleles *rpoE45* (W73R) and *rpoE47* (P118T) are included in Fig. 2A. When transferred into the Hfq mutant background together with the linked Δ*rseA*::Spc^R insertion, either *rpoE* mutation nearly abolished the increase of *eptB*::*lacZ* expression resulting from Hfq inactivation (Fig. 2A). This indicates that upregulation of *eptB*::*lacZ* in the Hfq mutant is for the most part σ^E-mediated. The slightly higher LacZ activity of the triple mutant, as compared with the RseA RpoE double mutant (Fig. 2A), might be ascribable to a σ^E-independent component in the regulation. It is noteworthy that, while the Δ*rseA*::Spc^R marker could be easily transduced into an Hfq$^-$ strain when linked to either *rpoE* mutation, no recombinants were obtained with wild-type *rpoE*. This suggests that the Δ*hfq* Δ*rseA* combination is lethal in an RpoE$^+$ background.

To further examine the involvement of σ^E in the Hfq mutant's phenotype, the effects of the *rpoE* mutations were analysed separately from the *rseA* deletion. Allele separation could be achieved only in the case of *rpoE45*. This mutation was rescued by transduction using a *cat* gene insertion on the 5′ side of the *rpoE* gene as selectable marker and identifying rare Spc^S recombinants. (Inheritance of the mutations was verified by DNA sequence analysis; the *cat* marker used for this experiment is part of a module replacing the *rpoE* promoter region described in more detail below). The *rpoE* alleles were subsequently moved into an Hfq$^-$ strain and β-galactosidase activity measured. Results in Fig. 2B confirm that *rpoE* mutations are epistatic to Hfq inactivation.

We anticipated that additional σ^E-controlled genes might be found among the fusions activated in the *hfq*$^-$ background. This was confirmed by the analysis of a sample of isolates which identified a subset of fusions similarly activated in the *rseA*$^-$ background. These include fusions to *rseC*, *ygaC*, STM1543 *sbmA*. The latter was previously identified in a search for σ^E-controlled genes in *E. coli* (Rezuchova *et al*., 2003). In contrast, *pduD*, *pduO*, *yifK*, *dppF*, *aphA* and *yihG* fusions remained largely unaffected (see Fig. 2C and D for representative examples). Surprisingly, *lamB* exhibited a peculiar behaviour, being nearly silenced in the presence of the *rseA* deletion. Mutations *rpoE45* and *rpoE47* reversed this effect, indicating that σ^E activity is responsible for the downregulation (Fig. 2E). Thus, the *lamB* response to *rseA* and *rpoE* mutations is opposite to that observed with the *eptB* fusion. It should be noticed that the experiment in Fig. 2E was carried out with cells growing in maltose-supplemented medium. Under these conditions, the

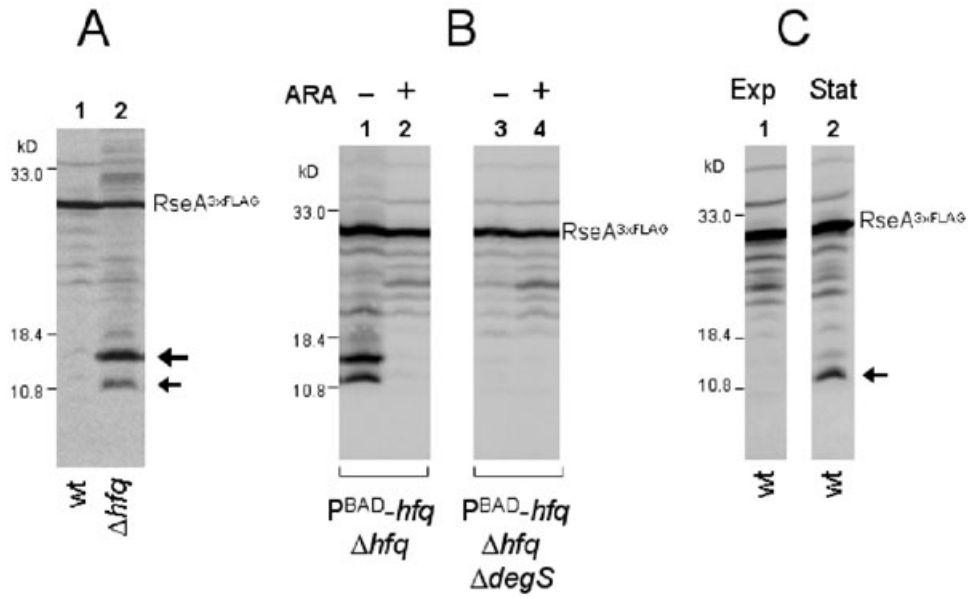

Fig. 3. Western analysis of 3xFLAG-tagged RseA protein. Overnight LB cultures were diluted 1:200 in fresh LB broth, and growth resumed at 37°C under aerobic conditions. In the experiment in B, overnight growth was in LB medium supplemented with 2 mM arabinose, and bacteria were rinsed twice in arabinose-free medium prior to dilution in LB with or without arabinose (2 mM). Cells were harvested when the culture reached an OD_{600} of 0.5, unless specified otherwise. Whole-cell extracts were fractioned in a 12% polyacrylamide-SDS gel which were processed for immunodetection of 3xFLAG-tagged proteins as previously described (Uzzau *et al*., 2002).
A. RseA pattern as a function of Hfq. Lane 1, strain MA8260 (*hfq*$^+$); lane 2, strain MA8311 (Δ*hfq*).
B. RseA pattern as a function of DegS and/or Hfq. Strain MA8312 (Δ*hfq* P^{BAD}-*hfq*) grown in LB medium without arabinose (lane 1) and with arabinose (lane 2). Strain MA8556 (Δ*degS* Δ*hfq* P^{BAD}-*hfq*) grown in LB medium without arabinose (lane 3) and with arabinose (lanes 4).
C. RseA pattern as a function of growth phase. Strain MA8260 (*hfq*$^+$) from a culture with OD_{600} = 0.5 (exponential) (lane 1) and from an overnight culture (stationary) (lane 2).

increase resulting from Hfq inactivation is less than measured in the absence of inducer (Fig. 1).

Loss of Hfq function elicits DegS-dependent RseA cleavage

One way to interpret the above data is to envisage that Hfq intervenes in the regulation of the *rpoE-rseABC* operon. The protein could bind to the 5′ end of the polycistronic transcript and, by selectively inhibiting translation of the first gene in the operon (*rpoE*), it could influence σ^E/RseA ratios in the cell. Alternatively, loss of Hfq function might upset the regulation of some membrane constituents so as to elicit an envelope stress response. To try distinguishing between these two scenarios, the entire region preceding the *rpoE* coding sequence was replaced with an heterologous module derived from the *lac* promoter (construct named $\Delta P^{rpoE}::P^{lac}$) and the effects of *hfq* inactivation on the expression of σ^E-regulated fusions (*eptB*::*lacZ*, *sbmA*::*lacZ*) measured in this background. Results showed the response of both fusions to be comparable to that of the strain carrying the native *rpoE* promoter configuration (Fig. 2B and data not shown). Thus, these experiments failed to substantiate a direct involvement of Hfq in *rpoE* gene regulation. We then tested the second hypothesis, namely that lack of Hfq activated the σ^E regulon indirectly, by generating an envelope stress condition. In *E. coli*, the σ^E response to envelope stress is initiated by the endoproteolytic cleavage of the RseA protein, carried out sequentially by the DegS and RseP proteases (Alba *et al*., 2002; Kanehara *et al*., 2002). We introduced a sequence encoding the 3xFLAG epitope at the 3′ end of the *rseA* gene. Crude extracts from bacteria expressing the carboxyl terminus-tagged protein were subjected to Western hybridization using anti-FLAG monoclonal antibodies. This analysis detected the presence, in all strains, of a protein whose size corresponded to that predicted for full-length RseA plus tag (27 kDa; Fig. 3). However, strains lacking Hfq also accumulated smaller products (particularly two bands in the 10–15 kDa range), absent or present in low amounts in the wild-type strain. In the strain carrying the *hfq* gene fused to the *ara* operon promoter, accumulation of the smaller products could be completely prevented by supplementing the growth medium with arabinose (Fig. 3B, lane 2). To confirm that these bands represented degradation intermediates, the analysis was repeated in a strain deleted for the *degS* gene [unlike in *E. coli*, DegS null mutants are viable in *Salmonella* (Rowley *et al*., 2005; see *Experimental procedures*)]. As shown in Fig. 3B (lane 3), the small-size bands were no longer detectable in such

Table 2. Bacterial strains.

Strain[a]	Genotype
TT10381	*hisD1284*::MudK *hisA9944*::MudI
SC1	*ompD159*::Tn*10*
SC4	*ompC390*::Tn*10*
MA3409	wild-type (Gifsy-1[-])
MA7455	wild-type (Gifsy-1[-])/pKD46
MA7791	*Δhfq67*::*cat* (CmR)
MA7872	*Δhfq67*::*catΔ*[*araBAD*]*68*::[*hfq aadA*] (P^{BAD}-*hfq*)
MA7892	*pduD1*::MudK
MA7893	*pduD1*::MudK*Δhfq67*::*cat*
MA7949	*pduO71*::MudK
MA7950	*pduO71*::MudK*Δhfq67*::*cat*
MA7953	*gudT76*::MudK
MA7954	*gudT76*::MudK*Δhfq67*::*cat*
MA8020	*yifK87*::MudK
MA8021	*yifK87*::MudK*Δhfq67*::*cat*
MA8022	*sbmA107*::MudK
MA8023	*sbmA107*::MudK*Δhfq67*::*cat*
MA8024	*aphA110*::MudK
MA8025	*aphA110*::MudK*Δhfq67*::*cat*
MA8026	*lamB111*::MudK
MA8027	*lamB111*::MudK*Δhfq67*::*cat*
MA8028	*eptB115*::MudK
MA8029	*eptB115*::MudK*Δhfq67*::*cat*
MA8108	*traT156*::MudK
MA8109	*traT156*::MudK*Δhfq67*::*cat*
MA8110	*ybfM172*::MudK
MA8111	*ybfM172*::MudK*Δhfq67*::*cat*
MA8119	*dppF181*::MudK
MA8120	*dppF181*::MudK*Δhfq67*::*cat*
MA8121	*dmsA186*::MudK
MA8122	*dmsA186*::MudK*Δhfq67*::*cat*
MA8146	*yihG441*::MudK
MA8148	*gabP543*::MudK
MA8149	*katE561*::MudK
MA8150	*hemN676*::MudK
MA8151	*rseC716*::MudK
MA8152	*ygaC721*::MudK
MA8154	*ego767*::MudK
MA8155	*mgtA774*::MudK
MA8167	Δ[P^{rpoE}]*69*::[*cat* P^{lacUV5}]
MA8180	*sbmA107*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}]
MA8182	*rseC716*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}]
MA8204	*eptB115*::MudK *rseA17*::Tn5-T-POP (TcR)
MA8260	*rseA71*::3xFLAG-*kan* (KnR)
MA8262	*Δ*[*rseA*]*70*::*aadA* (SpcR)
MA8311	*rseA71*::3xFLAG*kanΔhfq67*::*cat*
MA8312	*rseA71*::3xFLAG-*kanΔhfq67*::*catΔ*[*araBAD*]*68*::[*hfq aadA*] (P^{BAD}-*hfq*)
MA8385	*sbmA107*::MudK*ΔrseA70*::*aadA*
MA8386	*eptB115*::MudK*ΔrseA70*::*aadA*
MA8392	*sufE884*::MudK
MA8433	*yifK87*::MudK*ΔrseA70*::*aadA*
MA8471	*eptB115*::MudK*ΔrseA70*::*aadA rpoE45*
MA8472	*eptB115*::MudK*ΔrseA70*::*aadA rpoE47*
MA8498	*ΔdegS72*::*cat*
MA8511	*ΔdegS72*::scar
MA8516	STM1543::MudK
MA8521	*yihG441*::MudK*Δhfq67*::*cat*
MA8522	*hemN676*::MudK*Δhfq67*::*cat*
MA8523	*rseC716*::MudK*Δhfq67*::*cat*
MA8524	*ygaC721*::MudK*Δhfq67*::*cat*
MA8543	*ΔdegS72*::scar*Δhfq67*::*catΔ*[*araBAD*]*68*::[*hfq aadA*] (P^{BAD}-*hfq*)
MA8546	*lamB111*::MudK*ΔrseA70*::*aadA*
MA8555	STM1543::MudK*Δhfq67*::*cat*
MA8556	*rseA71*::3xFLAG-*kanΔdegS72*::scar*Δhfq67*::*catΔ*[*araBAD*]*68*::[*hfq aadA*] (P^{BAD}-*hfq*)
MA8621	*eptB115*::MudK*ΔmicA73*::*cat*
MA8622	*eptB115*::MudK*ΔmicF74*::*cat*
MA8623	*eptB115*::MudK*ΔmicC75*::*cat*
MA8624	*eptB115*::MudK*ΔmicA73*::*catΔrseA70*::*aadA*
MA8625	*eptB115*::MudK*ΔmicF74*::*catΔrseA70*::*aadA*
MA8626	*eptB115*::MudK*ΔmicC75*::*catΔrseA70*::*aadA*
MA8678	*gabP543*::MudK*Δhfq67*::*cat*
MA8679	*katE561*::MudK*Δhfq67*::*cat*
MA8680	*ego767*::MudK*Δhfq67*::*cat*
MA8740	*eptB115*::MudK *ompD159*::Tn*10*
MA8741	*eptB115*::MudK *ompD159*::Tn*10Δhfq67*::*cat*
MA8744	*lamB111*::MudK*ΔrseA70*::*aadA rpoE45*
MA8745	*lamB111*::MudK*ΔrseA70*::*aadA rpoE47*
MA8748	*sufE884*::MudK*Δhfq67*::*cat*
MA8783	*eptB115*::MudK *ompD*::Tn*10ΔrseA70*::*aadA*
MA8795	*eptB115*::MudK *ompD*::Tn*10ΔrseA70*::*aadAΔmicA73*::*cat*
MA8798	*eptB115*::MudK*Δhfq67*::*catΔrseA70*::*aadA rpoE45*
MA8799	*eptB115*::MudK*Δhfq67*::*catΔrseA70*::*aadA rpoE47*
MA8806	*eptB115*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}] *hfq13*::Tn5-T-POP
MA8814	*eptB115*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}] *rpoE45*
MA8816	*eptB115*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}] *rpoE45 hfq13*::Tn5-T-POP
MA8818	*eptB115*::MudKΔ[P^{rpoE}]*69*::[*cat* P^{lacUV5}]
MA8823	*eptB115*::MudK *rseA71*::3xFLAG-scar
MA8824	eptB115::MudK rseA71::3xFLAG-scarΔhfq67::cat

a. Except for strains TT10381 (Hughes and Roth, 1988) SC1, SC4 (Santiviago *et al*., 2003) and MA3409 (Figueroa-Bossi *et al*., 1997), all strains were constructed in this work, derived from MA3409. The latter is a derivative of *Salmonella enterica* serovar Typhimurium strain LT2 cured for the Gifsy-1 prophage (Figueroa-Bossi *et al*., 1997).

background. Taken together, these data strongly suggest that lack of Hfq in *Salmonella* activates the RseA proteolytic cascade. Releasing σ^E from inhibition results, among others, in increased transcription of the *rpoE-rseABC* operon (Skovierova *et al*., 2006), and *de novo* synthesis of RseA. This can explain why, in spite of extensive proteolysis, the full-length form of the RseA protein is present in the Hfq mutants to a level comparable to that of Hfq$^+$ strains. To determine whether presence of the C-terminal tag affected the function of the RseA protein, the regulation of the *eptB*::*lacZ* fusion was analysed in the background of the tagged construct (strains MA8823 and MA8824; Table 2). This work showed no significant change in the basal level of expression of *eptB*::*lacZ*, nor in the response to Hfq inactivation (data not shown). Thus, the epitope tag does not appear to affect neither, the RseA ability to inhibit σ^E under normal growth conditions, or RseA susceptibilty to DegS cleavage in the Hfq mutant.

RseA cleavage also occurs in stationary cultures of wild-type Salmonella

In the course of the above study, it became apparent that RseA cleavage products could be detected at low

level in wild-type bacteria whenever overnight cultures were used for the analysis. This prompted us to examine the role of growth phase on the cleavage pattern. Equivalent numbers of cells from exponential and stationary cultures of a wild-type strain expressing 3xFLAG-tagged RseA protein were lysed and used for Western analysis. As shown in Fig. 3C, the sample from stationary phase includes an approximately 11 kDa protein which is absent in the preparation from the exponential culture. Therefore, to a lesser extent than in the Hfq mutant, some RseA cleavage does occur in wild-type cells upon entry in stationary phase. Conceivably, the σ^E molecules released upon such cleavage become available for transcription. This might account for the observed activation of some σ^E promoters (see below).

σ^E-mediated downregulation of OMPs

Activation of the σ^E regulon in cells lacking Hfq might reflect an alteration in the synthesis of one or more envelope proteins. In *E. coli*, overexpression of porin OmpC is known to activate the σ^E response (Mecsas *et al*., 1993; Walsh *et al*., 2003). Expression of major OMPs OmpA, OmpC and OmpF is negatively controlled by small RNAs (MicA, MicC and MicF) through a mechanism involving destabilization of the transcripts (Mizuno *et al*., 1984; Chen *et al*., 2004; Rasmussen *et al*., 2005; Udekwu *et al*., 2005). Because Hfq is required for this activity, as well as for the stability of Mic sRNAs, we postulated that lack of Hfq might cause OMPs to be overproduced. This idea was tested by analysing the patterns of proteins from outer membrane preparations in polyacrylamide gels. To avoid band crowding problems due to the presence of a major *Salmonella* OMP (OmpD), which migrates between OmpA and OmpF (Santiviago *et al*., 2003), the study was carried out in strains with a disrupted *ompD* gene. Results in Fig. 4 show similar OMP patterns in wild-type and Hfq$^-$ strains, the only difference being the slightly higher OmpC levels in the mutant. We tested whether this increase were responsible for σ^E-activated phenotype of the Hfq mutant by measuring *eptB*::*lacZ* expression in a derivative carrying a Tn*10* transposon insertion in the *ompC* gene. Disrupting *ompC* did not affect *eptB*::*lacZ* regulation to any significant extent (data not shown), tentatively ruling out this porin as the source of the σ^E-activating signal in Hfq$^-$ cells. Strikingly, OmpA, OmpC and OmpF fell to nearly undetectable levels in the strain carrying the *rseA* deletion (Fig. 4, lane 3).

To complement the above data, strains were subjected to Northern analysis with probes complementary to *ompA*, *ompC* and *ompF* mRNAs (Fig. 5). Surprisingly, we found the levels of each of three mRNAs to be actually lower (anywhere between 20% and 50%) in the Hfq mutant as compared with wild type. A more efficient translation of these mRNAs might explain why this decrease does not have repercussions at the protein level. As predicted from the protein gel, the *rseA* deletion causes *ompA*, *ompC* and *ompF* mRNA levels to drop sharply. The effect is suppressed in the presence of *rpoE45* and/or *rpoE47* mutations, indicating that σ^E activity is responsible for the decrease. Activation of the σ^E response in the Hfq mutant might also underlie the decline of *omp* mRNAs in this mutant, because under conditions where such activation is impaired (Δ*degS*), loss of Hfq causes *omp* mRNAs to rise rather than decrease (compare lanes 8 and 9 in Fig. 5). To gain further insight, the RNA preparations were analysed with probes against the three Mic sRNAs (Fig. 6). This analysis revealed that the *rseA* deletion causes MicA RNA to accumulate during exponential growth, a phase in which the molecule is undetectable in wild-type cells. In contrast, the levels of MicC and MicF sRNAs are unaffected by the *rseA* mutation. These data strongly suggest that σ^E is involved in the transcription of the *micA* gene and directly responsible for activation of this gene in stationary phase. Failure to observe MicA sRNA accumulation in the RpoE RseA double mutants (Fig. 6, lane 4) and in the DegS mutant (Fig. 6, lane 6) further corroborates this idea. The disappearance of MicC and MicF sRNAs in the Hfq mutant is consistent with the notion that binding by Hfq protects sRNAs from

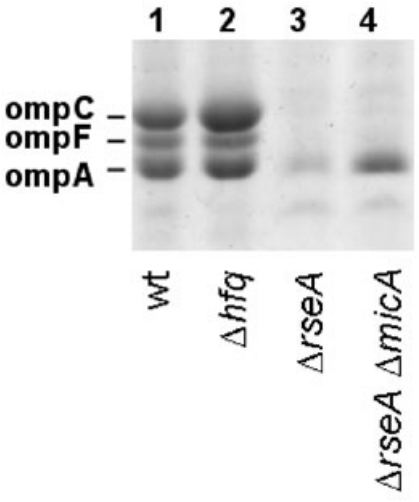

Fig. 4. Effect of *hfq* and *rseA* mutations on the levels of OmpC, OmpF and OmpA in the outer membrane of *Salmonella enterica* serovar Typhimurium. Cultures were grown in rich medium (LB; 5 g NaCl per litre) to an OD_{600} of 0.35. Outer membrane proteins were prepared as in Santiviago *et al.* (2003) and fractionated by SDS-PAGE on a 13% acrylamide gel. Only the portion of the gel with the bands corresponding to the major porins is shown. Lane 1, strain MA8740 (*ompD*::Tn*10*); lane 2, strain MA8741 (Δ*hfq ompD*::Tn*10*); lane 3, strain MA8783 (Δ*rseA ompD*::Tn*10*); lane 4, strain MA8795 (Δ*rseA* Δ*micA ompD*::Tn*10*). The complete genotypes of the strains used are in Table 2.

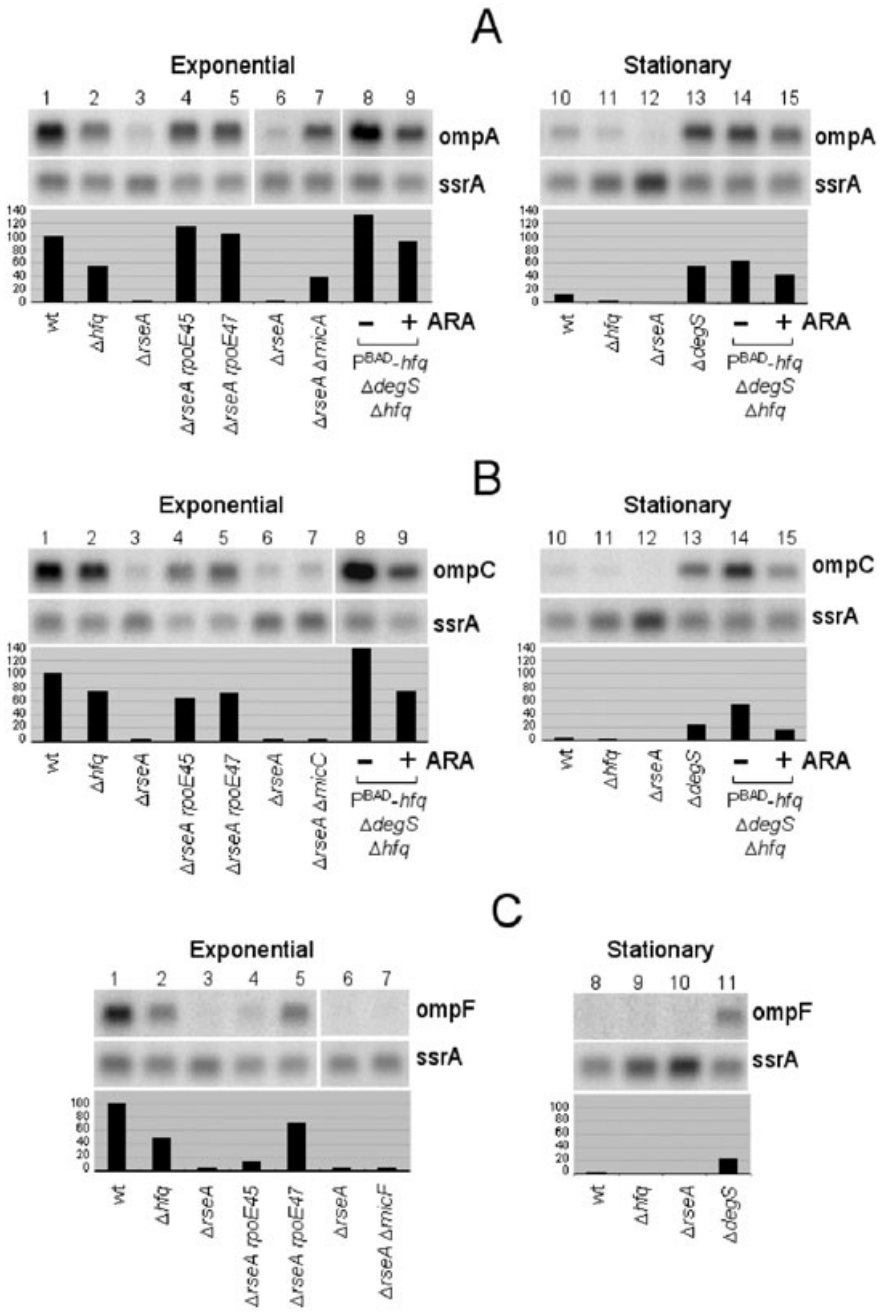

Fig. 5. Northern analysis of *omp* mRNAs. Cultures grown overnight in LB medium were diluted 1:200 in fresh LB and incubated with shaking at 37°C. RNA was extracted as previously described (Figueroa *et al.*, 1991) at $OD_{600} = 0.35$ (exponential) and $OD_{600} = 1.5$ (stationary). RNA was fractionated on 1.3% agarose-formaldehyde gels and transferred to Hybond-N^+ membranes, which were probed with radioactively labelled oligonucleotides complementary to *ompA* mRNA (A) *ompC* mRNA (B) and *ompF* mRNA (C). Each hybridization mixture contained a second probe complementary to SsrA (tmRNA). *omp* signals (quantified by phosphorimaging) were normalized to the corresponding *ssrA* signals, and values expressed as percentage of the value measured in the wild-type strain.

A. RNA from bacteria growing exponentially (lanes 1–9) or in stationary phase (lanes 10–15) was hybridized with oligonucleotides pp920 and pp813 (Table 4). Strains analysed were: MA8028 (wt) (lanes 1 and 10), MA8029 (Δ*hfq*) (lanes 2 and 11), MA8386 (Δ*rseA*) (lanes 3, 6 and 12), MA8471 (Δ*rseA rpoE45*) (lane 4), MA8472 (Δ*rseA rpoE47*) (lane 5), MA8624 (Δ*rseA*Δ*micA*) (lane 7), MA8543 (pBAD-*hfq*,Δ*degS*Δ*hfq*) grown in the absence of arabinose (lanes 8 and 14) and in the presence of arabinose (lanes 9 and 15), MA8498 (Δ*degS*), lane 13.

B. RNA obtained as in A was hybridized with oligonucleotides pp891 and pp813. Samples were loaded in the same order as in A except in lane 7 (MA8626;Δ*rseA*Δ*micC*).

C. RNA obtained as in A was hybridized with oligonucleotides pp892 and pp813. Strains used were: MA8028 (wt) (lanes 1 and 8), MA8029 (Δ*hfq*) (lanes 2 and 9), MA8386 (Δ*rseA*) (lanes 3, 6 and 10), MA8471 (Δ*rseA rpoE45*) (lane 4), MA8472 (Δ*rseA rpoE47*) (lane 5), MA8625 (Δ*rseA*Δ*micF*) (lane 7), MA8498 (Δ*degS*) (lane 11).

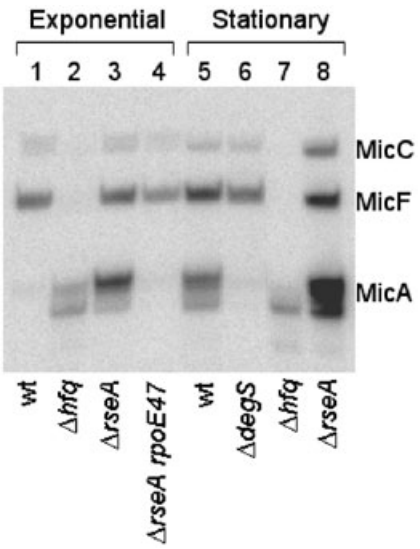

Fig. 6. Northern analysis of *mic* sRNAs. RNA extracted from bacteria growing exponentially (lanes 1–4) or in stationary phase (lanes 5–8) was fractionated on a 8% polyacrylamide-8 M urea gel and electrotransferred onto Hybond-N^+ membrane. The RNA blot was hybridized to a mixture of three oligonucleotide probes (pp814, pp831 and pp832; Table 4) complementary to MicC, MicF and MicA sRNAs respectively. (The relative positions of the three sRNAs in the gel were determined in experiments with single probes.) Strains used were: MA8028 (wt) (lanes 1 and 5); MA8029 (Δ*hfq*) (lanes 2 and 7), MA8386 (Δ*rseA*) (lanes 3 and 8), MA8472 (Δ*rseA rpoE47*) (lane 4), MA8498 (Δ*degS*) (lane 6).

degradation. In contrast, an RNA species hybridizing to the MicA probe still accumulates in the mutant, even though the banding pattern suggests increased decay (Fig. 6). Apparently, the higher transcriptional rate of the *micA* gene resulting from σ^E activation, offsets the increased degradation of the gene product. To test whether MicA was responsible for the disappearance of *ompA* mRNA in the RseA mutant, we examined the effects of deleting the *micA* gene. As shown in Fig. 5A (lane 7), the *micA* deletion causes the levels of *ompA* mRNA in the RseA mutant to increase significantly, although without reaching wild-type levels. In a similar trend, the *micA* deletion alleviates the downregulation of *ompA* mRNA in stationary phase but not to the same extent as a *degS* deletion (data not shown). Significantly, the effects of the *micA* deletion are also clearly detectable at the protein level (Fig. 4, lane 4) In summary, MicA sRNA contributes in the σ^E-mediated downregulation of *ompA* mRNA, but it does not seem to be the sole factor involved.

The levels of *ompC* and *ompF* mRNA vary in response to σ^E activation or inhibition in a way that closely resembles that observed for *ompA* (Fig. 5). However, the two Mic sRNAs previously known to regulate *ompC* and *ompF* mRNA levels (MicC and MicF, respectively) do not appear to participate in the σ^E-dependent regulation. Neither of these sRNAs is affected by the *rseA* deletion, nor by any of the changes of σ^E activity (*rpoE* point mutations, *degS* deletion) that were analysed in this study (Fig. 6). Furthermore, unlike what was observed with *micA*, deleting *micC* and *micF* genes does not affect *ompC* and *ompF* mRNA levels in the RseA mutant to any significant extent (Fig. 5B and C, lane 7). These data suggest the existence of additional σ^E-regulated sRNAs which control *omp* mRNA turnover in stationary phase.

Discussion

While the number of small RNAs identified in *E. coli* and other bacteria has risen dramatically in recent years, the roles of many of these molecules remain unknown. In the present study, we sought to identify potential targets of regulatory sRNAs in *Salmonella*, by isolating *lacZ* fusions to genes whose expression varied in the presence or absence of Hfq, the main sRNA chaperon. The majority of isolates obtained were downregulated by Hfq. Most of them carry the *lacZ* gene fused to genes for envelope constituents, including ABC transporters and porins. While some of these genes remain candidates for being under sRNA control, our work revealed that for others the response to Hfq is indirect, reflecting activation of σ^E-dependent transcription. The existence of an Hfq-σ^E connection was recently unveiled in *Vibrio cholerae* by Ding *et al*. (2004) who found that σ^E controls approximately half of the genes upregulated in the Hfq mutant. These authors mostly focused on the role of Hfq in *V. cholerae* pathogenicity and did not elucidate the basis for the protein's link with the σ^E response. Here, we have presented evidence showing that induction of the σ^E regulon in Hfq-deficient cells results from activation of RseA cleavage by the DegS protease. The actual signal initiating the proteolytic cascade remains unidentified. However, the high incidence of envelope genes among those found upregulated in the Hfq mutant suggests that activation may result from the accumulation of one or more envelope proteins. Overproduction of porins or other OMPs could cause some of these proteins to fold improperly and accumulate in the periplasm, leading to DegS activation (Mecsas *et al*., 1993; Walsh *et al*., 2003). Our experiments tentatively ruled out a contribution from major OMPs (LamB, OmpA, OmpC and OmpF) to the inducing signal. We found the genes for these proteins to become strongly downregulated during the σ^E response. Although this mechanism is impaired in the Hfq mutant, it is active enough to prevent the increase of *ompA*, *ompC* and *ompF* mRNA levels. (Such an increase does occur in the Hfq DegS double mutant where the σ^E response cannot be induced; see Fig. 5 in *Results*).

Fig. 7. Comparison of the DNA sequence at the 5′ side of the *micA* gene of *E. coli* and *S. enterica* with the conserved motifs of σ^E-dependent promoters. The position of the transcription start site in *E. coli* was determined by Udekwu *et al.* (2005). The consensus sequence for σ^E-dependent promoters (in the WebLogo format) is from Skovierova *et al.* (2006). The regions of sequence identity between *E. coli* and *S. enterica* are in shaded boxes.

Downregulation of *omp* genes is an interesting aspect of the σ^E response. The phenomenon was recently detected in *E. coli* and the possibility that it might involve σ^E-regulated sRNAs was considered (Rhodius *et al.*, 2006). As discussed in more detail below, the phenomenon is not confined to occasional stress conditions; rather, it is an integral part of growth phase-dependent control of *omp* genes. The most compelling evidence for this implication stems from the study of *ompA*. Previously, two independent studies identified MicA (SraD) as the main regulator of *ompA* mRNA levels. MicA sRNA accumulates in stationary phase and, upon binding to Hfq, interacts with a sequence at the 5′ end of *ompA* mRNA promoting the degradation of this RNA (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). The data presented in this article show that MicA accumulation requires a functional σ^E protein and its release from RseA inhibition. The *micA* promoter, identified by Udekwu *et al.* (2005) in *E. coli*, matches the recently defined consensus motifs recognized by σ^E (Rhodius *et al.*, 2006; Skovierova *et al.*, 2006) particularly at the most conserved positions (Fig. 7). The corresponding sequence in *Salmonella* is identical to the *E. coli* sequence in the conserved motifs and around the transcription initiation site (Fig. 7). It thus appears highly likely that the *micA* gene is transcribed by σ^E and activated upon entry into stationary phase. Stationary phase-induced activation of the σ^E response was reported previously but the mechanism remained undetermined (Testerman *et al.*, 2002; Bang *et al.*, 2005). Our data strongly suggest that activation results from DegS-mediated RseA proteolysis. First, DegS was required to observe accumulation of MicA sRNA in stationary cultures; second, specific RseA cleavage products were detected in cells entering stationary phase. From the low abundance of these species, one expects only a limited number of σ^E molecules to be released. Nonetheless, they may be sufficient to activate transcription from promoters showing high affinity for the sigma factor. Indeed, our study suggests a hierarchy in stationary-phase responses of different promoters; while the *micA* gene became rapidly transcribed, the *sbmA*::*lacZ* fusion, also a member of the σ^E regulon, did not respond to the growth phase to any significant extent. It thus appears that some σ^E promoters require full induction of the envelope stress response to be activated.

Deleting the *micA* gene in RseA mutant cells partially relieves the downregulation of *ompA,* raising mRNA levels to about 40% of wild-type (Fig. 4A, lane 7). Residual regulation could be ascribable to a different sRNA acting redundantly with MicA. Involvement of additional, σ^E-controlled sRNAs can also be invoked to interpret the data from other OMPs. We showed that neither MicC or MicF intervene in the σ^E-mediated control of *ompC* and *ompF* mRNA levels and yet these genes, as well as the *lamB*::*lacZ* fusion, respond to the *rseA* mutation in a way that closely parallels the *ompA* response and is similarly dependent on Hfq function. A recent report identified a new sRNA, RseX, which downregulates both *ompA* and *ompC* mRNA levels when overproduced from a multicopy plasmid in *E. coli* (Douchin *et al.*, 2006). The *rseX* gene was found to be totally silent in its natural chromosomal context (Douchin *et al.*, 2006). We checked whether the *Salmonella rseX* homologue could become expressed in *rseA* mutants, but were unable to detect the RseX sRNA in all strains and under all conditions tested. Nonetheless, the discovery of RseX points to the existence of additional sRNAs regulating *omp* mRNAs turnover, predicting that other molecules with this function will be identified in the near future.

Experimental procedures

Strains and growth conditions

Strains used in this study were all derived from *S. enterica* serovar Typhimurium strain LT2 (Table 2). Bacteria were cultured at 37°C in liquid media or in media solidified by the addition of 1.5% (w/v) Difco agar. Luria–Bertani broth (1% bacto tryptone (w/v), 0.5% Difco yeast extract (w/v), 0.5% NaCl (w/v)) was used as complex medium. Medium E supplemented with 0.2% (w/v) glucose or no-carbon E medium (NCE) supplemented with 0.2% (w/v) lactose were used as minimal media (Maloy, 1990). MacConkey-lactose indicator plates (Difco) or LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal; from Sigma), 40 μg ml^{-1}, were used to monitor *lacZ* expression in bacterial colonies. When required, antibiotics (Sigma) were included at the following final concentrations: chloramphenicol, 10 μg ml^{-1};

kanamycin monosulphate, 50 μg ml^{-1} (100 μg ml^{-1} in minimal medium) sodium ampicillin 50 μg ml^{-1}; spectinomycin dihydrochloride, 80 μg ml^{-1}; tetracycline hydrochloride, 25 μg ml^{-1}. Liquid cultures were grown in New Brunswick gyrotory shakers and growth was monitored by measuring the optical density at 600 nm with a Milton-Roy Spectronic 301 spectrophotometer.

Plasmids and transposons

Plasmids pKD3, pKD46 (Datsenko and Wanner, 2000) pSUB11 (Uzzau *et al*., 2001), pSEB5, pSEB5-*hfq* and pNFB21 (this work) were used in experiments involving l Red-mediated recombination. Plasmid pSEB5 is an oriR6K plasmid carrying the *lacZ* a-complementation fragment and multiple cloning site (MCS) from plasmid pUC8 near a spectinomycin-resistance, *aadA* gene. Plasmid pSEB5-*hfq* was derived from pSEB5 by cloning the *hfq* coding sequence into the XmaI site of the MCS. In pSEB5-*hfq*, the *hfq* gene lies upstream of the *aadA* gene and points in the same direction. Plasmid pNFB21 is an oriR6K plasmid carrying a *lacUV5* promoter-*lacZ* gene fusion and a *cat* gene, side-by-side in divergent orientation. Transposon Tn5-T-POP is a composite element comprising the core region of T-POP (Rappleye and Roth, 1997) flanked by the ends of transposon Tn5. MudK (original name: MudII1734) is a transposition defective Mu prophage that produces *lacZ* gene fusions upon inserting within coding regions (Castilho *et al*., 1984).

Enzymes and chemicals

Restriction enzymes, T4 polynucleotide kinase and Tac DNA polymerase were from New England Biolabs, Pfu-Turbo DNA polymerase was from Stratagene, T4 DNA ligase was from USB. A 3:1 mix of Taq/Pfu polymerase was used for the polymerase chain reaction (PCR). Oligonucleotides used as PCR primers or hybridization probes (obtained from Sigma Aldrich) are listed in Tables 3 and 4. Acrylamide-bisacrylamide (30%, 29:1) and other electrophoresis reagents were from Bio-Rad. Agarose was from Invitrogen. Anti-FLAG and anti-HA monoclonal antibodies as well as horseradish peroxidase (HRP) conjugated goat anti-mouse IgG were from Sigma.

Genetic techniques

Transduction was carried out using a high-frequency generalized transducing mutant of phage P22 as described by Maloy (1990). Gene replacement and epitope tagging were performed by the 'λ Red' recombination method (Datsenko and Wanner, 2000; Yu *et al*., 2000) following the protocols of Datsenko and Wanner (2000) and Uzzau *et al*. (2001; 2002) respectively. DNA fragments were generated by PCR using primers listed in Table 3. Strain MA7455, which expresses the λ Red recombinase from the pKD46 plasmid was used as host for transformation. Recombinant clones were verified by transduction and/or by PCR analysis combined with DNA sequencing. Mutants were routinely reconstructed by transferring the modified region into a clean background by P22-mediated transduction. For Tn5-T-POP mutagenesis,

Table 3. Oligonucleotides used as PCR primers for gene replacement.

Primer[a]	Sequence (5′-3′)	Template	Allele
pp560 (fw)	GAAAGGTTCAAAGTACAAATAAGCATATAAGGAAAAGAGAA*TGGAGAAAAAAATCACTGG*	pKD3	Δ*hfq67::cat*
pp561 (rv)	ATAAACAGCGCGTGAACTTATTCAGTCTCTTCGCTGTCCT*GGAACTTCGGAATAGGAACT*		
pp586 (fw)	TGTTTCTCCATACCTGTTTTTCTGGATGGAGTAAGACGAT*GGCTAAGGGGCAATCTTTAC*	pSEB5-*hfq*	Δ[*araBAD*]*68::*[*hfq-aadA*]
pp501 (rv)	TTTGCGTAGATAGTGTTTATCCAGCAGGGATTGCTGCATG*CGGCTTGAACGAATTGTTAG*		
pp714 (fw)	ACAGGACAGTTCAGGAGTTGTCATCATGGTTCTTGTTTAA*TGTAGGCTGGAGCTGCTTCG*	pNFB21	Δ[P^{proE}]*69::*P^{lacUV5}
pp715 (rv)	CCCGTTCAACCAGGACCTGGTCCGTTAACTGCTCGCTCAT*AGCTGTTTCCTGTGTGTCCA*		
pp755 (fw)	AGGCGTTGACGATAGCGGGATACTGGAAAAGGTATTAGGC*ATGCGCTCACGCAACTGGTC*	pSEB5	Δ*rseA70::aadA*
pp756 (rv)	CTAGCCGCCACAAGGGACATGGCAAACCAAAGTTGCTTCA*TTATTTGCCGACTACCTTGGTGA*		
pp757 (fw)	ACAGGTGCCAGGAATCCAAACTTTAGGAACGCAATCGCAG*GACTACAAAGACCATGACGG*	pSUB11	*rseA71::*3xFLAG
pp758 (rv)	TAGCCGCCACAAGGGACATGGCAAACCAAAGTTGCTTCA*TATGAAATATCCTCCTTAG*		
pp761 (fw)	TGAAGCTCTTACGTTCGGTCGCAATAGGTTTAATTGTCGG*CATATGAATATCCTCCTTAG*	pKD3	Δ*degS72*
pp762 (rv)	TATTCCTGCACCGTCACCTGGAACGTGAGCTGCTTATCAT*TGTAGGCTGGAGCTGCTTCG*		
pp875 (fw)	ATAAACTGAACTCTTTGTTCCGGGGCGAGTCTGAGTATATC*ATATGAATATCCTCCTTAG*	pKD3	Δ*micA73*
pp876 (rv)	CGAGCCGTTTGCCGCGTGGCTTGCAAAACACGCCTGAC*CCTGTAGGCTGGAGCTGCTTCG*		
pp877 (fw)	TGATAAAACAGAATCTTCATTCGCAACTAAAATAGTGAC*CCATATGAATATCCTCCTTAG*	pKD3	Δ*micF74*
pp878 (rv)	TAGTTTTAGGCAGGTAGCATAAATCAGCCGGGTGTGTCTG*TGTAGGCTGGAGCTGCTTCG*		
pp879 (fw)	AAAATTATACAATATCATTCGGTTACGATATTCTACGCCT*CATATGAATATCCTCCTTAG*	pKD3	Δ*micC75*
pp880 (rv)	ACCCGGCGCAGATTAAAAAATATTCTAAGGATTAACCTGG*TGTAGGCTGGAGCTGCTTCG*		

a. Primers are defined as 'forward' (fw) or 'reverse' (rv) depending on whether they have same or opposite orientation (5′-3′) as the gene being modified. The portions of primers annealing to template plasmids are shown in italics.

Table 4. Oligonucleotides used as probes for Northern analysis.

Primer	Sequence (5′-3′)	RNA target
pp813	GCGGAGGCTAGGGAGAGAGG	*ssrA*
pp814	ATGATGATAACAAATGCGCG	*micA*
pp831	AGGTTAACGCAATGGCCCAG	*micC*
pp832	AGGGGTAAACAGACATTCAG	*micF*
pp891	GCAGAGCTGGTACCAGGAGGGACAGTACTTTAACTTTCAT	*ompC*
pp892	ATCACCGCTGCCAGGATTTTGCGCTTCATCATTATTTATTACCCTC	*ompF*
pp920	CGCTACGGTAGCGAAACCAGCCAGTGCCACTGCAATCGCGATAGCTGTCT	*ompA*

bacteria were transformed with a complex of transposon DNA bound to cognate transposase (Epicentre Biotechnologies). The preformed complex was a generous gift from Duncan Parsons and Fred Heffron, OHSU, Portland, Oregon. Transformation was by electroporation using a Bio-Rad Micro-Pulser under the conditions specified by the manufacturer. MudK transposition was performed as described by Hughes and Roth (1988).

Beta-galactosidase assays

Activity of β-galactosidase was measured in toluene-permeabilized cells as described by Miller (1992) and is expressed in Miller units. Bacteria (grown in LB) were centrifuged and resuspended in saline solution prior to the assay.

Western analysis

For detection of 3xFLAG tagged proteins, bacteria growing in LB cultures were harvested by centrifugation and processed as previously described (Uzzau *et al*., 2001; 2002). Proteins separated by SDS polyacrylamide gel electrophoresis were transferred to polyvinylidene difluoride (PVDF) membranes and probed with anti-FLAG M2 monoclonal antibodies from Sigma. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma) was used as a secondary antibody. Detection was performed by Enhanced Chemioluminescence (ECL, Amersham) in a FUJI LAS3000 Lumi-Imager.

RNA extraction and Northern analysis

RNA was prepared by the acid-hot-phenol method from exponentially growing (OD_{600} of 0.35) or stationary cultures in LB medium as previously described (Figueroa *et al*., 1991). LB was supplemented with arabinose (2 mM final) when indicated. Total RNA was estimated from the value of the OD at 260 nm. For Northern analysis, 7.5 μg of total RNA were separated under denaturating conditions either by 8 M urea-8% polyacrylamide in TBE (Tris-borate-EDTA pH 8.3) buffer or by 2.2 M formaldehyde – 1.3% agarose gel electrophoresis in MOPS ([N-morpholino] propanesulphonic acid-sodium acetate-EDTA pH 7.0) buffer. For acrylamide gels, transfer of the RNA onto Hybond-N^+ membrane (Amersham), was performed with a semidry electrotransfer apparatus (Transblot SD; Bio-Rad); in the case of agarose gels, transfer to the same support was performed using a vacuum blotter (Boekel/Appligene) after mild denaturation treatment in 50 mM NaOH, 10 mM NaCl. RNA was crosslinked to membrane by UV irradiation (Statagene UV Stratalinker 2400). Membranes were hybridized to probes as follows: 5 pmol of oligonucleotide (Table 4) were 5′ end-labelled using 10 U of T4 polynucleotide kinase (New England Biolabs) and 30 μCi of [γ-^{32}P]ATP (3000 μCi mmol^{-1}, Amersham). Unincorporated radioactivity was eliminated by passage through Micro-Bio Spin 6 chromatography columns (Bio-Rad). Hybridization was carried out in Ambion Oligonucleotide Hybridization Buffer at 45–50°C following Ambion's protocol. RNA was analysed by Phosphorimaging using ImageQuant software.

Construction of relevant mutants

All constructs were made by the λ Red method (PCR primers in Table 3). A mutant carrying an *hfq* deletion (MA7791) was obtained upon replacing the region between the start (ATG) and stop (TAA) codons of the *hfq* gene with the coding sequence of the *cat* gene. The swap was designed to avoid undesired polarity effects on downstream genes. In MA7791, the *cat* gene is expressed from signals normally devoted to *hfq* expression. To construct a strain carrying an inducible *hfq* gene, the structural portion of the arabinose operon (*araBAD* genes) was exchanged with the *hfq-aadA* segment from plasmid pSEB5-*hfq* (see above). The exchange was designed to have the *hfq* coding sequence starting at the position normally occupied by *araB (*the downstream *aadA* gene being expressed from autonomous signals). Moving the *ara*::*hfq* fusion into *hfq* deletion strain MA7791 by P22 transduction yielded strain MA7892, in which the sole copy of the *hfq* gene is under the control of the *araBAD* promoter (P^{BAD}-*hfq*). An *rseA*-deleted strain (MA8262) was obtained by replacing the coding sequence of the *rseA* gene with the corresponding segment from the spectinomycin resistance *aadA* gene. In MA8262, the *aadA* gene is expressed from signals normally devoted to *rseA* expression. A $DegS^-$ mutant was constructed by exchanging the *degS* gene with the *cat* gene. To avoid possible deleterious effects resulting from lack of σ^E activity, the construction was initially performed in a RseA mutant. Subsequent transduction experiments confirmed the *degS* deletion to be perfectly tolerated in a wild-type background (Rowley *et al*., 2005). Finally, in strain MA8167, the *lacUV5* promoter replaces the region upstream from the *rpoE-rseABC* operon.

Isolation of lacZ *gene fusions to Hfq-regulated genes*

A phage P22 lysate prepared on strain TT10381 (kindly donated by Kelly Hughes, University of Utah, Salt Lake City)

was used to transduce strain MA7892 (see above) selecting kanamycin resistance (Kn^R). Strain TT10381 harbours a transposition-defective Mu (*lac* Kn^R) prophage (MudK) that can be transiently complemented by a second Mu prophage during transduction (Hughes and Roth, 1988). Transductants were selected either on E medium plates containing 0.2% (w/v) glucose or on LB plates (kanamycin was present throughout the experiment at a concentration of 50 μg ml^{-1} in complex media and of 100 μg ml^{-1} in minimal media). Colonies were replica-plated sequentially on LB plates supplemented with Xgal (40 μg ml^{-1}) and either 0.2% (w/v) glucose or 0.2% (w/v) arabinose. In some experiments, replica plating was performed on MacConkey plates supplemented with 1% lactose with or without 0.2% arabinose. Colonies exhibiting a colour difference in the presence or absence of arabinose were picked and purified by repeated streaking on selective medium. P22 lysates were prepared on these isolates and used to transduce two strains in parallel: MA3409 (wild-type) and MA7791 (Δ*hfq*) selecting Kn^R on Lac indicator plates. In all instances where transductants showed a colour difference between the Hfq^+ and Hfq^- pair, chosen clones were purified and used for further analysis. Not more than 10% of isolates initially identified by the arabinose screen maintained the colour phenotype in the second screen. The reasons for such a disparity were not investigated.

Identification of transposon insertion sites

The sequences surrounding the sites of MudK and Tn5-T-POP insertions were amplified by inverse PCR as described (Hartl and Ochman, 1996) and resulting fragments were subjected to automated DNA sequencing.

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Chapter III

Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica* (submitted)

Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica*

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Summary

Prokaryotic regulatory small RNAs act by a conserved mechanism and yet display a stunning structural variability. In the present study, we used mutational analysis to dissect the functional anatomy of RybB, a σ^E-dependent sRNA, that regulates the synthesis of major porins in *E. coli* and *Salmonella*. Mutations in the chromosomal *rybB* locus that altered the expression of an *ompC-lac* fusion were identified. Some of the mutations cluster within a seven-nucleotide segment at the 5' end of the sRNA and affect its ability to pair with a sequence 40 nucleotides upstream from *ompC* translation start site. Other mutations map near the 3' end of RybB, destabilizing the sRNA or altering its binding to Hfq. The 5' end of RybB is also involved in *ompD* regulation. In this case, the sRNA can choose between two mutually exclusive pairing sites within the translated portion of the mRNA. Some of the RybB 5' end mutations affect the choice between the two sites, resulting in regulatory responses that diverge from those observed in *ompC*. Further analysis of RybB target specificity identified *chiP* (*ybfM*), a gene encoding an inducible chitoporin, as an additional member of the RybB regulon. Altogether, our results indicate that an heptameric "seed" sequence is sufficient to confer susceptibility to RybB regulation.

Introduction

Non-coding small RNAs have emerged as relevant components of the regulatory repertoires of all living cells. Micro RNAs in eukaryotes and small regulatory RNAs in prokaryotes modulate gene expression at the post-transcriptional level through the establishment of base pair interactions with short complementary sequences in mRNA targets. In bacteria, sRNAs often pair with sequences immediately upstream of the translation start site or within the first few codons of the mRNA (Aiba, 2007, Gottesman, 2005, Vogel, 2009, Waters & Storz, 2009). Formation of the RNA duplex interferes with ribosome binding and inhibits translation initiation. Exposure of endoribonuclease cleavage sites in the untranslated mRNA stimulates its decay. The latter effect, although not essential for regulation, is thought to confer robustness to the process and to make it irreversible (Morita *et al.*, 2006). A notable variation of this theme is encountered in the inhibition of OmpD protein synthesis by MicC sRNA in *Salmonella*. MicC pairs with the sequence within the internal portion of *ompD* mRNA (codons 23-26) where formation of the RNA duplex does not affect ribosome binding nor progression; rather it acts by eliciting mRNA cleavage by RNAse E (Pfeiffer *et al.*, 2009).

Occasionally, sRNA pairing within the 5' untranslated region of an mRNA can prevent the target sequence from participating in a secondary structure that limits ribosomal access. In such cases, the interaction stimulates translation initiation and the sRNA thus acts as positive regulator (Frohlich & Vogel, 2009, Majdalani *et al.*, 2005). Interestingly, some sRNAs can downregulate certain genes while activating others. For example, RyhB, an sRNA synthesized under iron-limiting conditions, inhibits translation of several mRNAs encoding iron-storage proteins but activates translation of *shiA* mRNA which encodes a permease for shikimate, a siderophore precursor (Prevost *et al.*, 2007).

All mRNA-targeting sRNAs known to date perform their function complexed with chaperon protein Hfq. The latter plays the double role of protecting the sRNAs from degradation and stimulating their association with target mRNAs (Geissmann & Touati, 2004, Zhang *et al.*, 2002). A combination of sequence features and higher-order structures dictates the specificity of Hfq

binding (Brescia *et al.*, 2003, Zhang *et al.*, 2002). Consistent with the importance of Hfq in all sRNA-mediated regulatory mechanisms, *hfq* mutants show pleiotropic defects and are particularly susceptible to environmental stress (Brown & Elliott, 1996, Ding *et al.*, 2004, Figueroa-Bossi *et al.*, 2006, Guisbert *et al.*, 2007, Sittka *et al.*, 2007).

Unlike transfer RNAs, the other major class of "small RNAs" in living cells, which all conform to a strictly conserved construction plan, bacterial regulatory sRNAs stand out for their seemingly erratic domain organization. Some sRNAs pair with mRNA targets through their 5' ends (Bouvier *et al.*, 2008, Guillier & Gottesman, 2008) while others have their pairing domain internally (Geissmann & Touati, 2004, Prevost *et al.*, 2007) or even adjacent to the transcription termination hairpin (Figueroa-Bossi *et al.*, 2009, Rasmussen *et al.*, 2009). Lack of a common architecture, combined with the elusive nature of the Hfq binding determinant, makes it difficult to predict the sequence elements required for sRNA activity. Identification of such elements can profit from the analysis of regulatory mutants in which sRNA activity is impaired (Bossi & Figueroa-Bossi, 2007, Figueroa-Bossi *et al.*, 2009). In the present study, this mutational approach was used to dissect the functional anatomy of RybB, a small RNA that downregulates the synthesis of several outer membrane proteins (OMPs) in *E. coli* and *Salmonella* (Vogel, 2009). Initially identified in *E. coli* cells from stationary cultures (Wassarman *et al.*, 2001), RybB was later found to be under the control of alternative sigma factor σ^E and to accumulate under conditions causing or mimicking envelope stress (Johansen *et al.*, 2006, Papenfort *et al.*, 2006, Thompson *et al.*, 2007). RybB targets include the mRNAs for major outer porins, such as OmpC, OmpD, OmpN and OmpW (Bouvier *et al.*, 2008, Papenfort *et al.*, 2006). By preventing excessive accumulation of these proteins, RybB plays an important role in the maintenance of envelope homeostasis. An in vitro study of the RybB interaction with *ompN* mRNA showed that the initial 16 nucleotides of RybB sRNA form a RNA duplex with the portion of the mRNA spanning from the second to the seventh codon (Bouvier *et al.*, 2008). Although neither the Shine-Dalgarno sequence nor the initiation codon were included in the duplex, the structure effectively prevented binding of the ribosome to the mRNA (Bouvier *et al.*, 2008).

To further characterize RybB and to study its action at additional targets, we designed a genetic screen for randomly induced mutations that altered the ability of RybB to downregulate *ompC* and *ompD* mRNAs. Characterization of these mutants confirmed the role of the 5' sRNA portion in target recognition, as well as the contribution of specific residues to the selectivity and strength of the regulation. Mutations in the central portion of RybB identified residues involved in Hfq binding. A change near the 3' end of the sRNA revealed a role of the transcription termination hairpin in RybB sRNA function.

Results

The genetic screen

Mutants of RybB were generated through a procedure that combines mutagenic PCR with lambda red recombination (Bossi & Figueroa-Bossi, 2007). Chromosomal recombinants were screened in a *Salmonella* strain carrying a copy of the rpoE (σ^E) gene under the control of the chromosomal P^{BAD} promoter, and an *ompC-lacZ* fusion whose regulatory range lies within the sensitivity window of MacConkey indicator medium (see Materials and Methods). Mutant candidates, identified by their altered colony color on MacConkey-lactose plates supplemented with arabinose, were purified and analyzed by DNA sequencing. This analysis identified isolates carrying mutations in the chromosomal *rybB* locus. These mutants fell into two general classes. One class carried changes in the *rybB* promoter region that cause the -35 or the -10 motifs to depart from the consensus sequence of σ^E promoters (Fig. 1S). These mutations presumably relieve *ompC* repression by lowering *rybB* transcription and were not further analyzed. A second class of mutants, the majority in our study, contained changes within the structural portion of the *rybB* gene. These mutations cluster at three separate locations, corresponding to the 5’ end, the center, and the 3’ end of the sRNA (Fig. 1A). The 5’ end mutations mapped within the segment previously shown to base pair with *ompN* mRNA (Bouvier *et al.*, 2008).

In addition, the mutagenic PCR approach was applied to a DNA region extending about 500 bp on either side of the *ompC* AUG start codon. Mutants were screened for increased *ompC-lac* expression as above. Most of the mutations obtained map upstream from the main *ompC* promoter. These changes increase *ompC* transcription from either the main promoter or from a second weaker promoter further upstream (data not shown). Two mutations, however, fall within the 5' untranslated region (5'UTR) of *ompC* mRNA, within a 10-nucleotide sequence complementary to the 5' end of RybB sRNA (Fig. 1A). One of the two alleles, *ompC* U38A, is predicted to restore base pairing in the presence of the *rybB* allele A4U. Strains carrying either one or both of these mutations were constructed, and *ompC* expression was quantified. The results in Fig. 1B show that *ompC* U38A and *rybB* A4U individually cause *ompC* expression to increase. However, their association in the same strain restores repression. This confirms that the repression mechanism requires a base pair interaction. The observation that *ompC* U38A affects regulation to a greater extent than *rybB* A4U might be explained by a greater difficulty to accommodate an A:A mismatch, as opposed to a U:U in the duplex structure (Du *et al.*, 2005). The second *ompC* mutation, A31G, causes an A:U base pair to be replaced by a G:U base pair. This mutant is more difficult to interpret given the conservative nature of the change and its lateral position in the duplex. Perhaps the A31G change antagonizes RybB pairing indirectly, by affecting the size of a secondary structure on the 5’ side of the duplex region (see Fig. 3S). The portion of the RybB sequence that pairs with *ompC* mRNA is the same previously shown to interact with *ompN* mRNA (Bouvier *et al.*, 2008). However, while in the latter and in other RybB-regulated mRNAs (see below), the target sequence lies within the coding region, the *ompC* target site is located in the 5' UTR at a distance from the start codon.

To extend the mutational analysis, RNA from *ompC* 5' UTR was synthesized in vitro and its interaction with *in vitro*-made RybB RNA or with purified Hfq protein was probed by footprinting techniques. The results in Fig. 1C show that RybB protects the segment between U29 and C39 of *ompC* mRNA from digestion by RNases A and T1, thus confirming the participation of this sequence in a RNA duplex. Moreover, the analysis indicates the presence of a secondary structure adjacent to the 5’ side of the pairing segment in

ompC mRNA. This structure does not appear to be disrupted by the formation of the RNA duplex, suggesting that pairing does not extend beyond the initial 10 nucleotides of RybB. Significantly, the portion of *ompC* mRNA protected by Hfq binding begins immediately to the 3' side of the pairing segment and extends as many as 40 bases downstream (Figs. 1A and 1C). The length of this segment suggests the presence of two or more Hfq binding sites. The entire region is A/U-rich and includes two AAUAA motifs proposed to constitute high affinity Hfq binding sites (Figueroa-Bossi *et al.*, 2009, Soper & Woodson, 2008). Interestingly, this region also includes the 16-nucleotide sequence recognized by MicC, a second *ompC*-downregulating sRNA (gray bar in Fig. 1A) (Chen *et al.*, 2004).

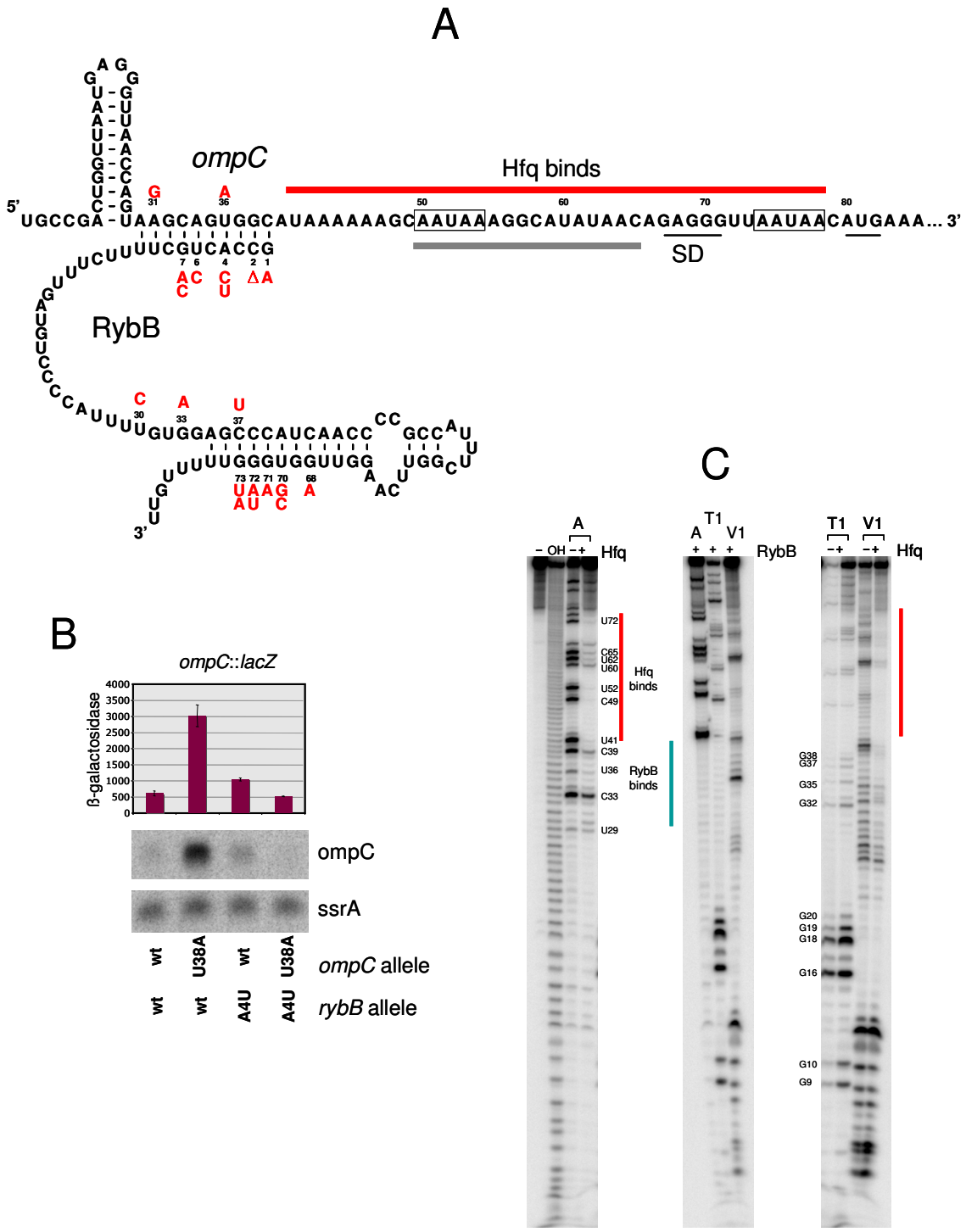


Fig. 1. *In vivo* and *in vitro* analysis of the *ompC*:RybB RNA interaction. **A.** Schematic representation of the pairing between *ompC* 5' UTR and RybB sRNA with the base changes that relieve RybB-mediated repression of *ompC*. Mutations were isolated following random PCR mutagenesis of the *rybB* gene, or of the *ompC* 5' UTR, and screening for altered expression of an *ompC-lac* fusion on lactose indicator plates (see text). A red bar above the sequence indicates the region protected by Hfq from RNAse cleavage (see below); a gray bar indicates the portion of the sequence that pairs to MicC sRNA (Chen *et al.*, 2004). Two AAUAA sequence motifs are boxed. The Shine-Dalgarno sequence and the AUG initiator codon are underlined. The 5' ends of *ompC* mRNA was mapped by primer extension (Fig. 2S). **B.** Effects of RybB/*ompC* compensatory changes on *ompC-lacZ* expression or on *ompC* mRNA levels in Northern blots. The strains employed for the LacZ assays were isogenic derivatives of strain MA9380 (*ompC94-lacZ ΔompD* P^{BAD}*-rpoE*; see Table 1). ß-galactosidase activity was measured from cultures grown in the presence of 0.2 % arabinose to stationary phase (OD600 = 2). The strains used for the Northern blot analysis were derived from strain MA8386 (*ΔrseA*; see Table 1 for full genotype). **C.** RNAse footprinting of *ompC* RNA complexes with Hfq or RybB sRNA. RNA was synthesized in vitro and ^{32}P-labeled at 5' end with T4 polynucleotide kinase. The *ompC* mRNA fragment corresponds to the +1 to + 104 portion of the

ompC transcript. RNA was incubated in the absence (-) or in the presence (+) of 100-fold excess of Hfq or 30-fold excess of in vitro made RybB sRNA for 30 min at 30°C (Hfq) or 37°C (RybB) prior to nuclease treatment. Red and green bars indicate the regions protected by Hfq and RybB, respectively.

The effects of *rybB* mutations on *ompC* and *ompD* regulation were assessed measuring ß-galactosidase activity in strains carrying *ompC::lacZ* and *ompD::lacZ* translational fusions or monitoring *ompC* and *ompD* mRNAs by Northern blot hybridization. The results from the group of alleles at the 5' end of the sRNA (the pairing mutants) are shown in Fig. 2A. Some of the mutations cause as little as a two-fold effect on *ompC* regulation, underlining the sensitivity of the genetic screen. The largest defect is observed in a mutant with a single base deletion at position 2 of the sRNA. The *ompC* and *ompD* expression levels in this strain (fivefold and fourfold higher than the repressed levels, respectively) approach those of a strain deleted for the entire *rybB* gene, indicating that the ΔC2 allele completely inactivates the sRNA. Interestingly, certain alleles affect *ompC* and *ompD* expression differentially. The G7A change, in particular, rather than relieving *ompD* repression, actually makes it tighter than in the wild-type. Such hyper-repression is reflected both in the ß-galactosidase activities and in the Northern blot analysis. Examination of the sequence on the 5' side of the *ompD* gene reveals two potential RybB pairing sites resulting from the tandem duplication of the six-base sequence AGUGGC in the region between the sixth and ninth codon of the mRNA (Fig. 2B). The G7A change is expected to increase the affinity of RybB sRNA for the site proximal to the AUG, as it replaces a G:U base pair with an A:U base pair. Thus these findings tentatively indicate that the proximal site is recognized by RybB. The question is then whether the distal site can also function as a RybB pairing sequence. To answer this question, identical mutations were introduced at corresponding positions of the two putative pairing sequences, as well as a compensatory change in RybB sRNA (Fig. 3A). The *ompD* G16C mutation allele does not have a significant effect on *ompD* expression while *ompD* G22C lowers expression below the wild-type level. Hyper-repression is also observed when either of these alleles is combined with compensatory change C5G in the sRNA (Fig. 3B). Thus, these results confirm that both sites are functional targets for RybB. As predicted, combining *ompD* G16C and *ompD* G22C results in the complete loss of RybB-mediated regulation

(Fig. 3B). Downregulation is restored by *rybB* C5G, supporting the involvement of base pairing in the regulatory mechanism. It should be noticed that the two *ompD* mutations result in the replacement of a valine codon (GUG) for a leucine codon (CUG). The fact that these two triplets are the most frequently used codons for the respective amino acids in *E. coli* (Hénaut & Danchin, 1996) rules out the possibility that changes in codon usage could have influenced the results.

Overall, the data in Fig. 3 suggest that the two pairing interactions are mutually exclusive. The hyper-repressive pattern resulting from changes that disrupt one or the other of the interactions is surprising; it might suggest that competition between the two sites interferes with RybB activity. This view is supported by the behavior of RybB mutation G7A, whose main effect is to weaken pairing at the distal site. We also notice that the G7A change causes a slight increase in RybB sRNA levels (Fig. 2A). This increase, observed with independent isolates of the G7A mutant, might contribute to hyper-repression. The basis for the increase remains unknown, but it might reflect changes in sRNA turnover.

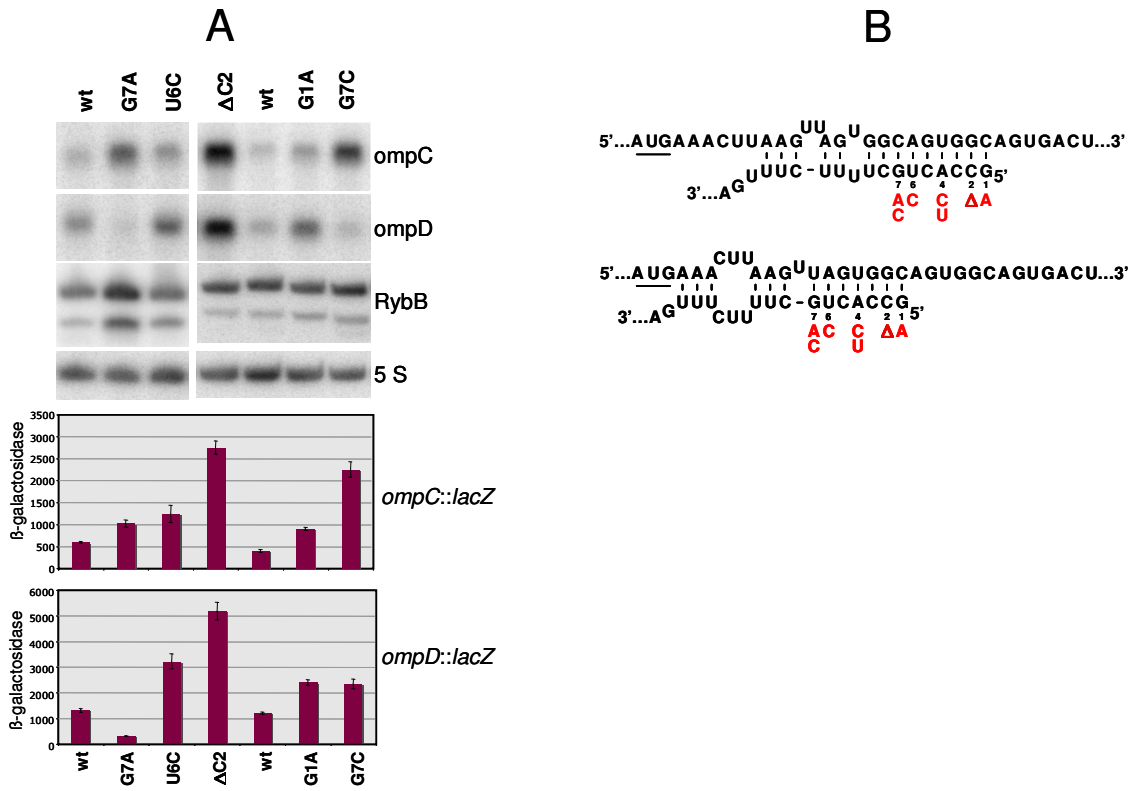


Fig. 2. Effects of *rybB* mutations on *ompC* and *ompD* regulation. **A.** Northern blot quantification of *ompC* and *ompD* mRNAs and ß-galactosidase activity in strains with translational *ompC-lacZ* and *ompD-lacZ* fusions. The Northern blot analysis was carried out with isogenic strains derived from MA8386 (*ΔrseA*). In the ß-galactosidase measurements, the *ompC-lacZ* carrying strains were derived from MA9380 (*ompC94-lacZ ΔompD PBAD-rpoE*) and the assays performed with cultures grown in the presence of 0.2 % arabinose. The *ompD-lacZ* strains were derivatives of MA9578 (*ompD96::lacZ ΔrseA*). All assays were carried out on cells grown to stationary phase (OD600 = 2). **B.** Alternative pairing interactions between RybB and the N-terminal coding portion of *ompD* mRNA. Occurrence of both types of interactions is supported by the results in Fig. 3.

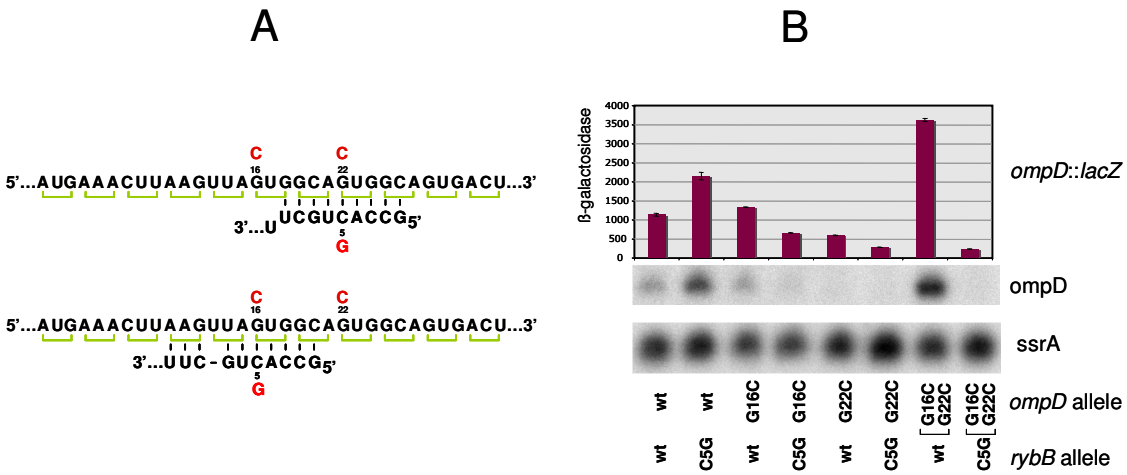


Fig. 3. Alternative RybB-pairing sites in *ompD* mRNA. Mutations were generated in the Salmonella chromosome by site-directed mutagenesis (see Materials and Methods). **A.** Alternative RybB:*ompD* pairing configurations and the base changes introduced in this study. *ompD* bases are numbered starting from the first base of the initiating AUG. **B.** Effects of *ompD* and/or *rybB* sequence changes on *ompD* expression. Expression was measured assaying ß-galactosidase activity in strains carrying the *ompD96::lacZ* fusion (top panel) and probing *ompD* mRNA in Northern blot (bottom panel). Strain genetic backgrounds and experimental protocols were those described in the legend to Fig. 2.

The Hfq binding mutants

All *rybB* mutations outside the pairing segment have similar effects on *ompC* and *ompD* regulation (Fig. 4; data not shown). With the exception of allele U70C (discussed below), these mutations cause RybB levels to decrease significantly, suggesting that they affect the stability of the sRNA rather than its activity (Fig. 4). Three alleles, U30C, G33A and C37U, affect nucleotides in the central portion of the sRNA near the bottom of the CG-rich stem loop structure that likely constitutes the Rho-independent transcription terminator (Fig. 1A). Since binding to chaperone protein Hfq is essential for RybB stability (our unpublished data), we speculated that the three mutations might lower the affinity of the sRNA for Hfq. To test this hypothesis, we compared the binding of wild-type and mutant sRNAs to purified Hfq in a gel mobility shift assay. Both U30C and G33A cause a small but reproducible reduction in the RybB affinity for Hfq (Fig. 5). The U30C allele shortens a run of four U residues, an alteration consistent with the known affinity of Hfq for poly U RNAs and A/U-rich tracks. In contrast, the defect of the G33A mutant is surprising given that the change increases the A/U richness in the region. The G33A position is at the hinge between a single-stranded region and the stem-loop terminator structure. Perhaps the change affects the relative orientations of these two domains in a way less favorable to the Hfq interaction. Footprinting experiments in Fig. 6 show that both the single stranded region near the basis of the terminator stem and the sequence of the 5' arm of the stem are weakly protected by Hfq, suggesting that both domains come in contact with the protein.

No significant alteration of Hfq binding could be detected in the C37U mutant (data not shown). This allele replaces a C:G base pair at the bottom of the terminator stem with an U:G pair. Although a conservative change, the position of the change might be critical for the overall stability of the structure. Destabilization of the terminator could in turn result in increased RybB turnover (see below).

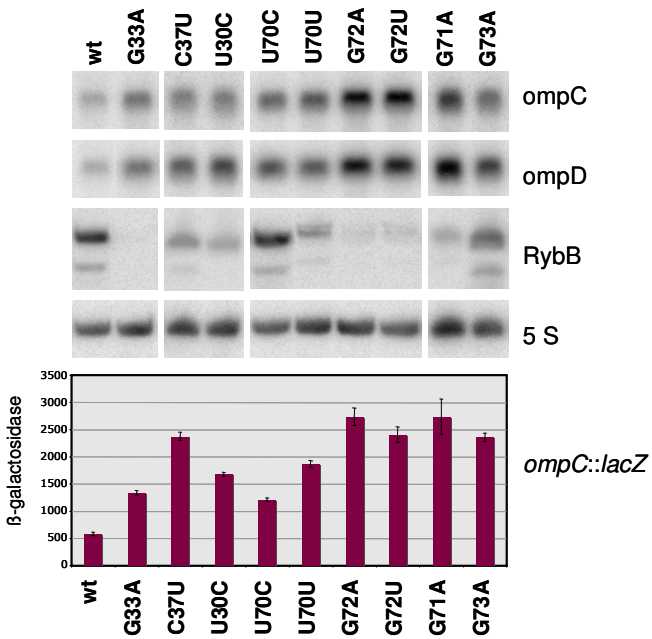
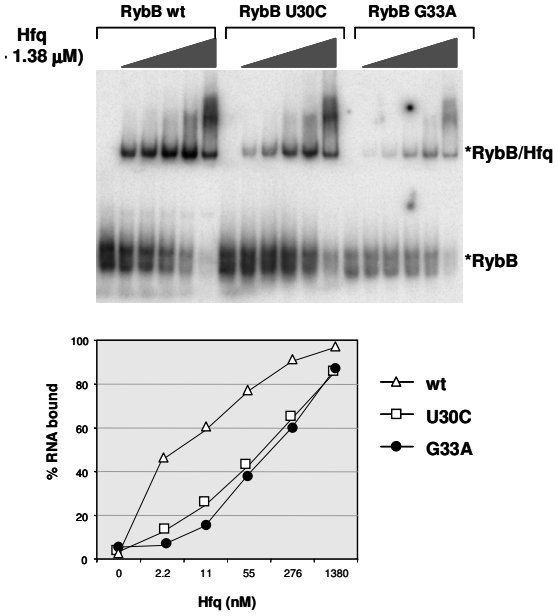


Fig. 4. Effect of *rybB* mutations on *ompC* and *ompD* expression and RybB sRNA stability. Northern blot analysis (top portion of Fig.) was carried out on RNA separated on a 1.2% agarose-formaldehyde gel (*ompC* and *ompD* probing) or on an 8% polyacrylamide-8 M urea gel (RybB and 5S RNA probing). Strains used were isogenic derivatives of MA8386 (Δ*rseA*). ß-galactosidase activity (histogram) was measured in strains derived from strain MA9380 (*ompC94-lacZ* Δ*ompD* P^{BAD}-*rpoE*) as described in the legend to Fig. 1.

Fig. 5. Effect of *rybB* alleles U30C and G33A on RybB:Hfq binding. **A.** Gel shift assay with purified Hfq protein. In vitro synthesized wild-type and mutant RybB RNA (4 nM), labeled at the 5′ end with ^{32}P, was incubated with increasing amounts of purified *Salmonella* Hfq protein (0, 2, 11, 55, 276, 1380 nM) under the conditions described in Materials and Methods. Samples were loaded on a 5% non-denaturing polyacrylamide gel and radioactivity quantified by phosphorimaging. **B.** Quantification of band intensity in the gel in A. Quantification was performed with Fuji Film Multi Gauge software.



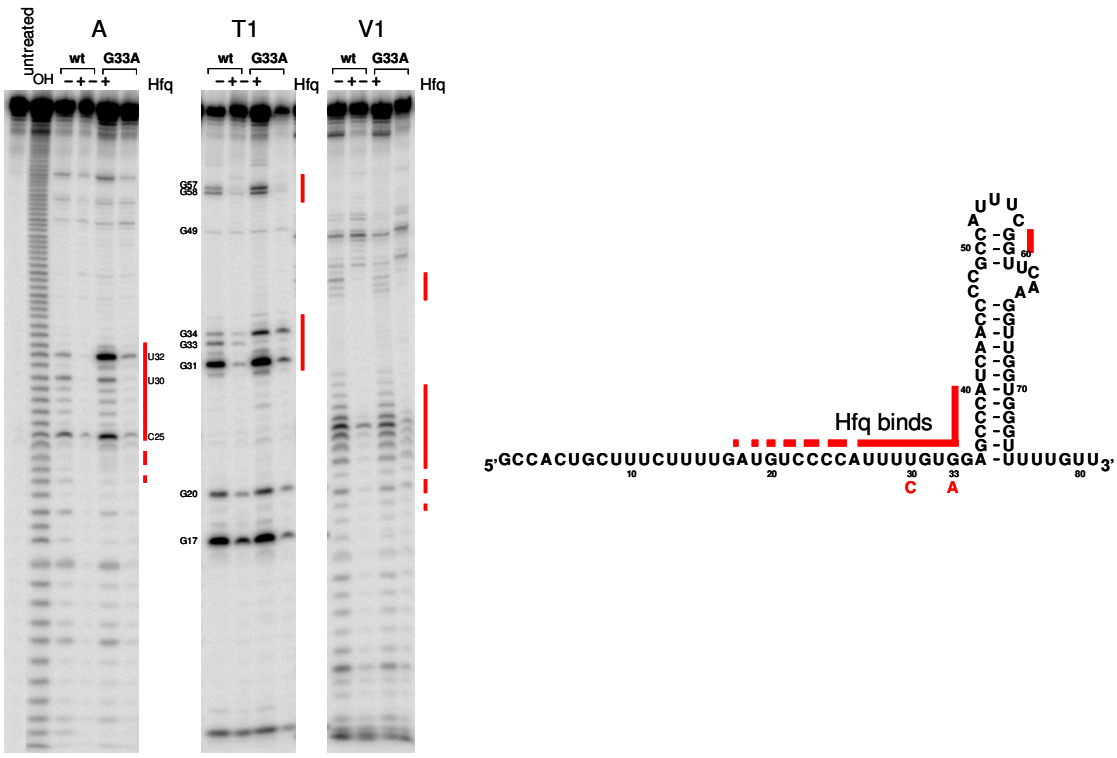


Fig. 6. Footprinting of RybB:Hfq complexes. **A.** In vitro made ^{32}P-labeled RybB^{wt} or RybB G33A mutant RNA, either alone or in the presence of 100-fold excess of Hfq, was incubated with the indicated ribonucleases for 12 min at 37 °C. Red bars indicate the regions protected by Hfq. **B.** Schematic representation showing the protected sequences in the RybB sRNA.

The terminator mutants

A last group of *rybB* mutations are clustered within a short segment corresponding to the 3' arm of the terminator stem. All these mutations disrupt base pairing in the lower portion of the stem and, with one exception, cause the partial or complete destabilization of the sRNA (Fig. 4). These findings support the notion that the terminator structure is a stabilizing element in prokaryotic RNAs (Aiba *et al.*, 1991, Guarneros & Portier, 1991, Mott *et al.*, 1985). Intriguingly, the U70C allele does not cause any detectable decrease in RybB sRNA levels (Fig. 4). To characterize this mutant, *in vitro* synthesized sRNA was subjected to ribonuclease probing. From the cleavage pattern in Fig. 7, it appears that U70C causes the terminator region to rearrange in an alternative configuration. In particular, the increase in the reactivity of G36, G57 and G58 to RNAase T1 and of U54 to RNAse V1 is consistent with a model in which the entire 5' arm of the stem slides in a 5' direction to allow a sequence in the apical portion of the arm (CCCGCC) to pair with the complementary segment

generated by the U70C change (GGCGGG) (see Fig. 7B). As a result, the terminator stem is shortened while the single-stranded region on its 5' side is extended. Somehow surprisingly, given the magnitude of the conformational change, gel mobility assays failed to reveal any alteration in Hfq binding (data not shown). Nonetheless, the latter findings are consistent with the *in vivo* stability of the mutant sRNA (Fig. 5). From the regulatory phenotype of the mutant, one would infer that the conformational rearrangement somehow hampers RybB ability to interact with its target sequences.

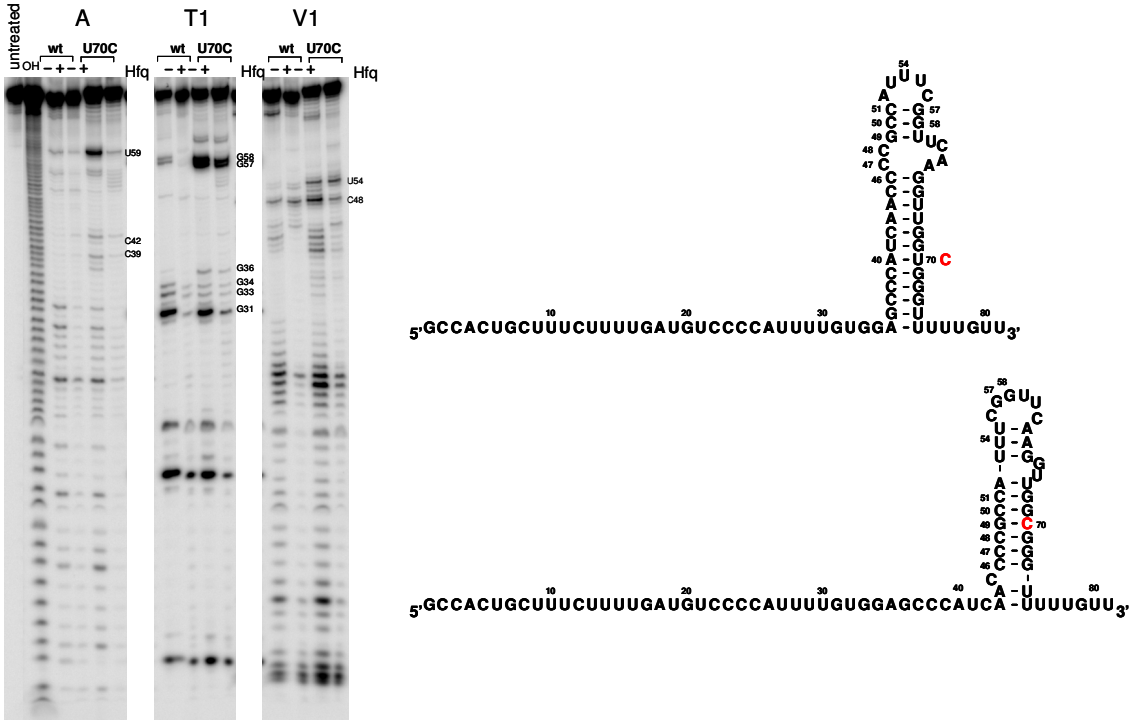


Fig. 7. Structural analysis of the RybB U70C mutant. **A.** *In vitro* made ^{32}P-labeled RybB^{wt} or RybB U70C mutant RNA, either alone or in the presence of 100-fold excess of Hfq, was incubated with the indicated ribonucleases for 12 min at 37 °C. **B.** Schematic representation showing the conformational rearrangement caused by the U70C change, as inferred from the reactivity patterns in A.

A new member of the RybB regulon

ChiP (YbfM) is an inducible porin that allows passage of chitin-derived oligosaccharides (chitobiose and chitotriose) across the outer membrane (Figueroa-Bossi *et al.*, 2009). ChiP synthesis is normally inhibited by constitutively made ChiX (MicM) sRNA, which pairs with *chiP* mRNA preventing translation (Figueroa-Bossi *et al.*, 2009, Rasmussen *et al.*, 2009). ChiX inhibition is relieved in the presence of chitosugars as a result

of the accumulation of an RNA species that pairs with ChiX sRNA and promotes its degradation (Figueroa-Bossi *et al.*, 2009, Overgaard *et al.*, 2009). The presence of a sequence complementary to the 5' end of RybB within the initial portion of the *chiP* coding sequence led us to test whether *chiP* expression was affected by *rybB* mutations. This analysis, carried out in strains deleted for the *chiX* gene, reveals increased *chiP-lacZ* expression in most *rybB* mutants (Fig. 8). In contrast, the RybB G7A allele, predicted to improve the pairing between RybB and *chiP* tightens *chiP-lac* repression (Fig. 8). The slight increase in RybB sRNA levels resulting from the G7A change (see above) might contribute to repression. Thus, these findings confirm that *chiP* is a target for RybB regulation. The relatively narrow range of the regulatory response (less than a 50% variation) might be attributed to the shortness of the *chiP*:RybB RNA duplex (limited to 7 base pairs).

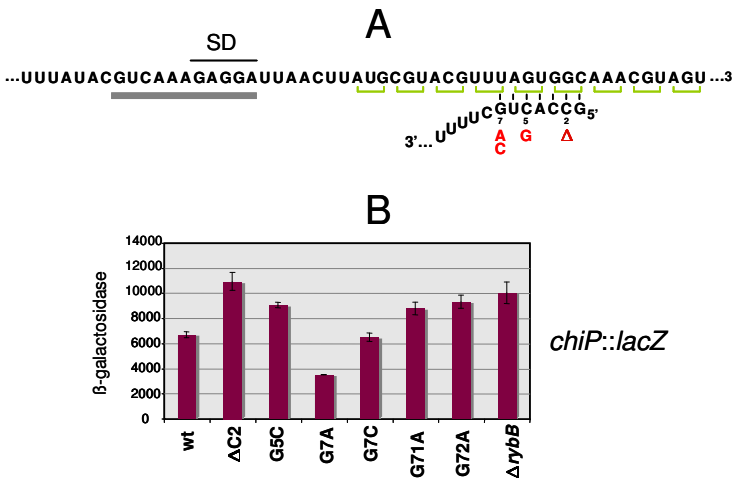


Fig. 8. Effect of *rybB* mutations on *chiP* expression. A. Model for pairing between RybB sRNA and the sequence near the start of the translatated portion of *chiP* mRNA. A gray bar indicates the sequence segment pairing with ChiX sRNA (Figueroa-Bossi et al., 2009). B. ß-galactosidase activity in strains carrying a translational *chiP-lacZ* fusion. Besides the indicated *rybB* alleles, strains used carried deletions of *chiX* and *rseA* genes (Table 1).

Discussion

RybB is a small non-coding RNA that is synthesized in response to σ^E activation and downregulates the expression of several major outer membrane proteins (Johansen *et al.*, 2006, Papenfort *et al.*, 2006, Thompson *et al.*, 2007). Because overaccumulation of OMPs is one of the signals leading to σ^E activation, RybB activity contributes to the maintenance of OMP homeostasis and serves as basis for autogenous control. Here we used a mutational approach to define the RNA sequence elements that participate in RybB-mediated regulation. The study was carried out entirely in the *Salmonella* chromosome and relied on a genetic screen sensitive enough to detect mutations causing as little as a two-fold effect in RybB-mediated regulation. The results identified the sequence of the 5' end of RybB as the main determinant of recognition of *ompC* and *ompD* target mRNAs. The same region had been previously shown to interact with *ompN* mRNA (Bouvier *et al.*, 2008), leading to the prediction that the 5' end of RybB would be involved in the regulation of additional targets (Vogel, 2009). A similar pattern was recently demonstrated for two redundant sRNAs with a wide target range, OmrA and OmrB, which also pair with their targets through their 5' ends (Guillier & Gottesman, 2008). Our genetic analyses indicate that the first 7 bases of the RybB sequence play the predominant role in the interaction with either *ompC* or *ompD* mRNAs. This finding is strongly reminiscent of postranscriptional regulation by eukaryotic miRNAs, where a 7-base "seed" region is the minimal requirement for regulation (Lewis *et al.*, 2003, Brennecke *et al.*, 2005). In *ompC* mRNA, a secondary structure near the 5' side of the pairing region limits the total length of the duplex to 10 base pairs or less. The pairing segment, 40 nucleotides upstream from the start codon, lies just outside the portion of the mRNA covered by the 30S subunit of ribosome during initiation (Huttenhofer & Noller, 1994). Still, formation of the RybB:*ompC* RNA duplex might sequester residues needed for efficient docking of the ribosome onto the mRNA and thus slow down translation initiation (Sharma *et al.*, 2007). Alternatively, formation of the duplex could stimulate cleavage of *ompC* mRNA by a ribonuclease (Massé *et al.*, 2003, Morita *et al.*, 2005). Footprinting experiments showed that Hfq binds the region

between the RybB paring sequence and the *ompC* AUG *in vitro*, consistent with the presence of two putative high-affinity binding sites (AAUAA) within the region. Intriguingly, the upstream motif is part of the sequence recognized by MicC sRNA (Chen *et al.*, 2004). This raises the possibility that conditions that stimulate MicC activity are incompatible with optimal (Hfq-assisted) RybB recognition of its target sequence. The second AAUAA motif lies in the interval between the Shine-Dalgarno sequence and the initiation codon. Hfq binding to this site might exert a direct effect on *ompC* mRNA translation, either contributing to obstruction of the ribosome entry site and/or causing *ompC* mRNA destabilization through the recruitment of RNAse activity (Morita *et al.*, 2005). This possibility is in agreement with genetic evidence indicating that the effects of *hfq* mutations on OMP homeostasis are partially independent of MicA and RybB sRNAs (Bossi *et al.*, 2008).

The study of *ompD* regulation showed the presence of two seed regions for RybB sRNA, one spanning codons 5 to 7, the other spanning codons 7 to 9. Surprisingly, mutations that affect pairing at either site increase repression, suggesting that the redundancy is somehow detrimental to RybB activity. Perhaps collisions can occur between RybB molecules trying to pair simultaneously at the two positions.

In the course of this study, we found that the chitoporin gene, *chiP*, is an additional RybB-downregulated gene in *Salmonella*. Synthesis of ChiP protein was recently shown to be specifically induced in the presence of chitosugars in the growth medium (Figueroa-Bossi *et al.*, 2009). The induction mechanism involves inactivation of a different sRNA, ChiX (MicM), which normally inhibits translation of *chiP* mRNA (Figueroa-Bossi *et al.*, 2009, Overgaard *et al.*, 2009). ChiP accumulates massively following induction (Figueroa-Bossi *et al.*, 2009). Therefore, it is not surprising that *chiP* may be also subject to the RybB-dependent homeostatic control as other major OMPs.

Supporting material

Fig. 1S. DNA sequence changes in *rybB* promoter mutants. The consensus sequence for σ^E-dependent promoters is from Skovierova *et al.*, Microbiology 152: 1347-1359 (2006).

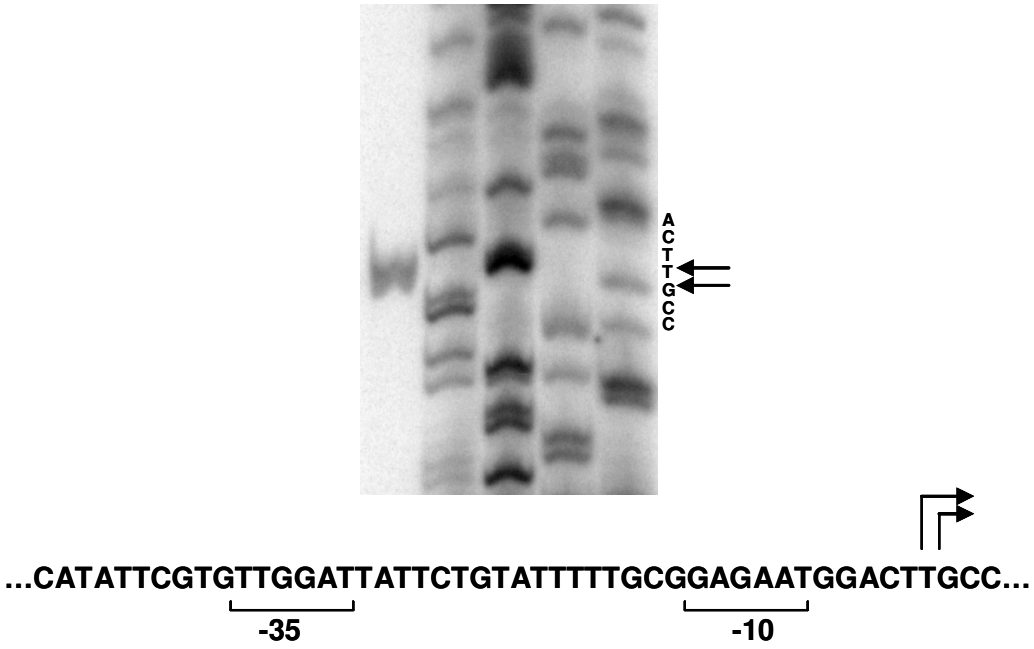


Fig. 2S. 5'-end mapping of *ompC* mRNA by primer extension. The reverse transcriptase reaction (Superscript II from Invitrogen) was carried out using 5 μg of RNA from strain MA3409 (wt) with primer ppA68 labeled at the 5' end with ^{32}P. The same primer was used to generate the DNA sequence latter. The sequencing template was a DNA fragment obtained by PCR amplification of chromosomal DNA with primers pp832 and ppA68. (For primer sequences see Table 2). Reactions were performed with the *fmol* DNA Cycle Sequencing System from Promega, according to the manufacturer's protocol.

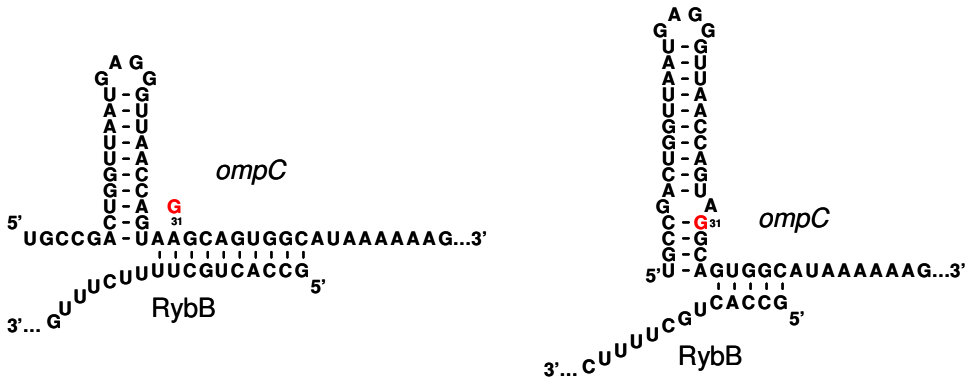


Fig. 3S. Model for how *ompC* mutation A31G could influence pairing with RybB sRNA. The A31G change might cause some of the bases normally involved in the RybB interaction (positions 30 through 34) to become part of an extended secondary structure to the 5' side of the pairing region.

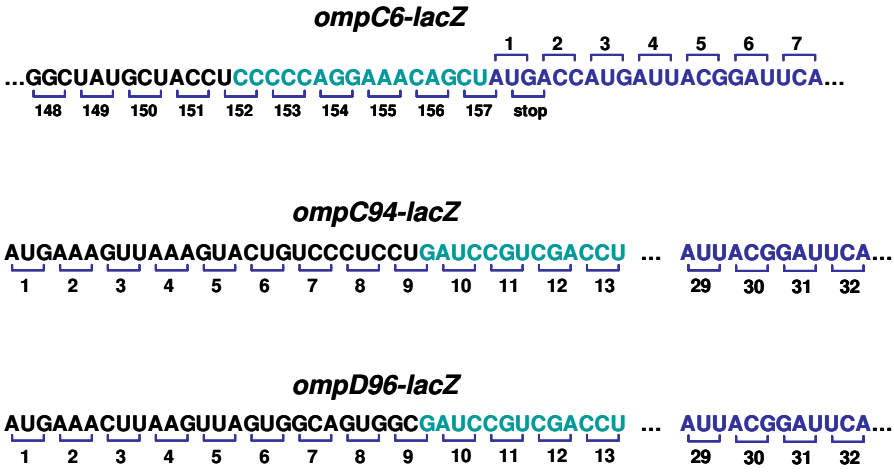


Fig. 4S. RNA sequences at the boundaires of the *lacZ* fusions used in this study. Fusions were obtained as described in Materials and Methods. The sequence segments in green precede the *lacZ* gene (in blue) in suicide plasmids pCE36 (*ompC6::lacZ*) or pCE40 (*ompC94::lacZ and ompD96::lacZ*) (Ellermeier *et al.*, Gene 290: 153-161 (2002).

Experimental Procedures

Strains and growth conditions

Strains used in this study were all derived from *Salmonella enterica* serovar Typhimurium strain MA3409 (Figueroa-Bossi *et al.*, 1997). The genotype of relevant strains used are shown in Table 1. Bacteria were cultured at 37°C in liquid media or in media solidified by the addition of 1.5% Difco agar. LB broth (Bertani, 2004) was used as complex medium. Carbon-free medium (NCE) (Maloy & Roth, 1983), supplemented with the appropriate carbon source, was used as minimal medium. Antibiotics (Sigma) were included at

the following final concentrations (in LB): chloramphenicol, 10 µg/ml; kanamycin monosulphate, 50 µg/ml; sodium ampicillin 75 µg/ml; spectinomycin dihydrochloride, 80 µg/ml; tetracycline hydrochloride, 25 µg/ml. LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; from Sigma), 40 µg/ml, were used to monitor lacZ expression in bacterial colonies. Liquid cultures were grown in New Brunswick gyrotory shakers and growth was monitored by measuring the optical density at 600 nm with a Milton-Roy Spectronic 301 spectrophotometer.

Enzymes and chemicals

Restriction enzymes, T4 polynucleotide kinase and Taq DNA polymerase were from New England Biolabs, Pfu-Turbo DNA polymerase was from Stratagene, T4 DNA ligase was from USB. DNA oligonucleotides were obtained from Sigma Aldrich. The sequences of the oligonucleotides used in this work are shown in Table 2. Acrylamide-bisacrylamide (30%, 29:1) and other electrophoresis reagents were from BioRad. Agarose was from Invitrogen. Hybond-N+ membranes and hybridization buffer used for Northern blot analysis were from GE Healthcare and from Applied Biosystems-Ambion, respectively. MegashortScript T7 kit, Kinase Max kit, RNAses A and T1, yeast tRNA were all from Applied Biosystems-Ambion.

Genetic techniques

Generalized transduction was performed using the high-frequency transducing mutant of phage P22, *HT 105/1 int-201* (Schmieger, 1972) as described (Lemire *et al.*, 2008). Chromosomal engineering was carried out by the λ red recombination method (Datsenko & Wanner, 2000, Yu *et al.*, 2000) as previously described (Uzzau *et al.*, 2001). Donor DNA fragments were generated by PCR using plasmid or chromosomal DNA templates. Amplified fragments were introduced into strains expressing phage λ red operon from plasmid pKD46 (Datsenko & Wanner, 2000). When required, the antibiotic-resistance cassette introduced by recombination was excised upon transforming strains with plasmid pCP20, which expresses the Flp

recombinase (Cherepanov & Wackernagel, 1995). Preparation of recipient bacteria, DNA electroporation and isolation and processing of recombinant clones were carried out as described (Lemire *et al.*, 2008).

Construction of relevant strains

The constructs described below were obtained by the λ red recombination method. DNA oligonucleotides used as primers for the PCR reactions are listed in Table 2. Template plasmids were pKD3, pKD4 and pKD13 (Datsenko & Wanner, 2000).

Construction of lac fusions

To readily identify chromosomal mutations affecting RybB-mediated regulation of *ompC*, at the beginning of this study, we sought to develop a reporter system suitable to reveal such mutants directly on lactose indicator plates. The strategy used involved: i) inserting a promoterless lac operon immediately downstream from the transcription terminator of the *ompC* gene in a strain with a chromosomal P^{BAD}-*rpoE* fusion; ii) selecting Lac+ derivatives resulting from terminator deletions fusing *ompC* to *lacZ* ; iii) identifying Lac+ colonies whose color on MacConkey lactose plates turned from red to pink when the medium was supplemented with arabinose. Insertion of the *lac* operon downstream from *ompC* was achieved by integrating an *aph* cassette [conferring kanamycin resistance (KnR), amplified from plasmid pKD4 with DNA oligonucleotide primers pp893 and pp894] followed by its Flp recombinase-mediated replacement with suicide plasmid pCE36 as described (Ellermeier *et al.*, 2002). Lac+ mutants were selected on NCE plates containing 0.2% lactose as the sole carbon source. For the colorimetric screen, Lac+ mutants were replica plated on MacConkey-lactose plates with or without arabinose. One of the isolates showing the color change was analyzed further. Sequence analysis revealed that the deletion, *ompC6::lac*, removes approximately two thirds of the *ompC* gene but does not generate an in-frame fusion. Rather, the *lacZ* initiator AUG codon falls right at the junction with *ompC* DNA (overlapping,

in -1 frame, a UGA stop codon at the join point), suggesting that *lacZ* expression results from translational reinitiation at the *ompC-lacZ* boundary (Fig. 4S). Finding that *ompC6::lac* responds to RybB regulation suggests that reinitiating ribosomes originate from the translation of the upstream *ompC* segment. The fusion served as basis for the screening of *rybB* mutants (see below).

For quantitative measurement of *ompC* or *ompD* gene expression, in-frame *lacZ* fusions were constructed. The procedure involved the insertion of the *aph* module of plasmid pKD4 in each of the genes, and Flp recombinase-mediated conversion of the insert to a lac fusion with plasmid pCE40 as described (Ellermeier *et al.*, 2002). The primers used for the amplification of the aph cassette were ppB22 and ppB23 for the *ompC* construct, and pp998 and ppA86 for the *ompD* construct. Primer extension sequences were chosen so as to place the fusion boundary within *ompC's* or *ompD's* signal peptide-encoding segments. Disruption of the signal sequence was intended to prevent deleterious effects that might result from translocation of the ß-galactosidase moiety of the hybrid proteins across cell membranes. In the final constructs, *ompC94::lac* and *ompD96::lac*, the *lacZ* coding sequence is fused to the 7th codon of *ompC* or *ompD* (Fig. 4S).

Other constructs

Chloramphenicol-resistance (CmR) markers linked to *rybB* or *ompC* genes were obtained by amplifying the cat module of plasmid pKD3 with the primers below, and using the resulting fragment for λ red mediated recombination. Insertion *Δ[STM0869]::cat* (primers ppA11 and ppA12) places the *cat* gene 55 base pairs (bp) downstream from *rybB* gene in parallel orientation. Allele *ΔyojN::cat* (primers ppB31 and ppB32) places the *cat* gene 507 bp upstream from the coding sequence of the *ompC* gene in opposite orientation. A chromosomal P^{BAD} promoter fusion to the σ^{E} (rpoE) gene (*Δ[araBAD]76::Tn5TPOP45 PBAD-rpoE*), obtained replacing the *araBAD* portion of the ara operon with the *rpoE* coding sequence was described in a previous study (Bossi & Figueroa-Bossi, 2007).

Random PCR mutagenesis

DNA fragments encompassing the region to be mutagenized and the linked cat marker (see above) were amplified by PCR under error-prone conditions (Bossi & Figueroa-Bossi, 2007). In the *rybB* mutagenesis, the template was chromosomal DNA from strain MA9147 (Table 1) and the primers ppA40 and ppA41 (Table 2). For the mutagenesis of *ompC* 5' UTR, template was chromosomal DNA from strain MA9242 (Table 1) and the primers were ppA62 and ppA63 (Table 2). In both amplification experiments, the priming sites were selected so as to have approximately 0.5 Kb on either end of the DNA products providing homology for recombination. The fragments obtained (2035 bp and 2043 bp, respectively) were used to transform strain MA9133 (*ompC6::lac* P^{BAD}*-rpoE*) and CmR recombinants selected on MacConkey-lactose plate supplemented with 0.2 % arabinose. Colonies appearing darker red than the background colonies were picked and purified by streaking on selective plates. The mutagenized region was PCR-amplified (ppA40/ppA8, RybB; pp832/ppA68 *ompC* 5' UTR) and subjected to DNA sequence analysis.

Site-directed mutagenesis

To obtain *rybB* allele C5G, a DNA fragment amplified from chromosomal DNA (strain MA9147) with primers ppD50 (which contains the desired base change) and ppA41 was introduced into strain MA9133 (Table 1) and CmR recombinants selected on MacConkey plates supplemented with 1% lactose and 0.2% arabinose. DNA from colonies with a darker red color was confirmed to contain the desired mutation. To introduce changes in the RybB pairing segment of *ompD* (alleles G16C, G22C and the double change), the procedure used was the same as in the isolation of the *aph* insertion in the signal sequence (above), except that forward primer was ppD47 in the making of G16C, ppD48 for G22C, and ppG24 to produce the double mutant. Reverse primer was ppA86 in all cases. Template was pKD13 plasmid DNA. Presence of the appropriate mutations in selected recombinant clones was confirmed by sequence analysis. Flp-mediated

excision of the *aph* insert restored the *ompD* reading frame downstream from the mutated sites. These constructs were used directly for *ompD* mRNA analysis or they were converted to *lacZ* fusions for ß-galactosidase determinations (see above).

RNA extraction and Northern analysis

RNA was prepared by the acid-hot-phenol method from exponentially growing cells (OD600 of 0.35) as previously described (Bossi & Figueroa-Bossi, 2007). RNA was separated on a 1.2% agarose-formaldehyde gel (*ompC* and *ompD* mRNA analysis) or on an 8% polyacrylamide-8 M urea gel (RybB sRNA analysis) and blotted onto a nylon membrane. Blots were hybridized to 5' end-labeled DNA oligonucleotide probes specific for the RNAs under study or for RNAs serving as internal standards (ssrA, 5S RNA). Oligonucleotides used were pp891 (*ompC*), pp931 (*ompD*, Figs 2 and 4) ppG25 (*ompD*, Fig. 3), pp929 (RybB), pp813 (*ssrA*) and ppB10 (5S RNA). The sequences of these probes are shown in Table 2. In the experiments in Figs 2 and 4, blots hybridized with pp891 were stripped of the label (2 x 15 min in hot 0.1% SDS) and re-hybridized with probe pp931.

Beta-galactosidase assays

Activity of β-galactosidase was measured in toluene-permeabilized cells as described by (Miller, 1992) and is expressed in Miller units. Typically, the activity was measured in 10 µl of cultures grown to stationary phase. Reported values were the average of at least two independent determinations, each involving duplicate or triplicate samples.

In vitro RNA synthesis

Templates for *in vitro* transcription with T7 polymerase were generated by PCR from genomic DNA. To produce the *ompC* 5’ UTR template, DNA was amplified with primers pp952 and pp953. To make the wild-type *rybB* template, primers were ppC46 and ppC47, while primers ppC46 and ppC57 were used to produce the *rybB* U70C allele (Table 2). *In vitro* transcription

was performed with the MegashortscriptT7 kit (Ambion AM1354) according to the manufacturer's protocol. After incubation for 2 hrs at 37°C, DNAse was added and incubation continued for further 15 min. The sample was treated with phenol and the RNA precipitated at -20°C overnight with sodium acetate-ethanol-glycogen. RNA was recovered by centrifugation, resuspended in water and quantified by nanodrop reading. The RNA solution was adjusted to 5 pmol/µl. RNA (10 pmols) was dephosphorylated and labeled at its 5' end with [γ^{32}P]ATP (3000 Ci/mmol from Perkin Elmer) using the KinaseMax kit (Ambion AM1520). Labeled RNA was purified by 8% PAGE. The RNA band was eluted from the gel, phenol-extracted, ethanol precipitated at -20°C and resuspended in water. Before use for Hfq binding or structural studies, RNA was heated in refolding buffer (50 mM Tris pH8, 0.1 M NaCl, 0.1 M KCl 1 mM MgCl2) at 85°C for 3 min, followed by 20 min at room temperature and then placed on ice.

Gel shift assays

7His-tagged *Salmonella* Hfq protein was purified as described (Figueroa-Bossi *et al.*, 2009). Labelled *ompC* 5' UTR or RybB RNA (4 nM), was incubated with increasing amounts of protein Hfq as indicated, in 50mM TrisHCl pH 7.8, 50 mM NaCl, 50 mM KCl, 10 mM MgCl and 6 µM yeast tRNA at 30°C for 30 min. Binding reactions were loaded on a 5% non-denaturing polyacrylamide gel. Electrophoresis was in 0.5 X TBE buffer at 4°C for 3 hrs at constant current of 15mA. Results were analyzed by phosphorimaging using ImageQuant software.

RNA Structural analyses

Enzymatic treatments were performed in 10 µl of reaction mix containing 0.2 pmols of RNA, 1 µg of yeast tRNA, 1x Structure buffer (Ambion) and 0.01 ng of RNase A (Ambion AM 2274, 1 mg/ml) or 0.1 U of RNase T1 (Ambion AM 2283, 1U/ml). Incubation was at 37°C for 12 min. Reactions were stopped by addition of 20 µl of completed "Precipitation/Inactivation" buffer from the same manufacturer. Partial alkaline hydrolysis was performed according to Ambion's protocol as follows: 10 µl of reaction mix

containing 0.2 pmols of RNA, 1 µg of yeast tRNA, 1x Alkaline Hydrolysis buffer, were incubated at 95°C for 8 min, placed on ice and 20 µl of completed Precipitation/Inactivation buffer added. After recovery from precipitation, all samples were run on a 10% sequencing polyacrylamide gel in 0.5x TBE. Results were analyzed by phosphorimaging.

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Table 1. Relevant *Salmonella* strains used in this work

Strain[a]	Genotype	Source or reference
MA3409	wild-type	(Figueroa-Bossi *et al.*, 1997)
MA7455	wild-type / pKD46	(Figueroa-Bossi *et al.*, 2006)
MA8262	*ΔrseA70*::*aadA* (SpcR)	(Figueroa-Bossi *et al.*, 2006)
MA8386	*eptB115*::MudK *ΔrseA70*::*aadA*	(Figueroa-Bossi *et al.*, 2006)
MA8685	Δ[*araBAD*]*76*::*rpoE* Tn*5*TPOP*45* (P^{BAD}-*rpoE*)	(Bossi & Figueroa-Bossi, 2007)
MA8775	*eptB115*::MudK Δ*rybB77*::*cat*	(Bossi & Figueroa-Bossi, 2007)
MA8904	*eptB115*::MudK Δ*ompD78*::*aadA*	(Bossi *et al.*, 2008)
MA8933	Δ[*chiX-ybaP*]::*cat*	(Figueroa-Bossi *et al.*, 2009)
MA9132	*chiP91*::pCE40(*lac*)	(Figueroa-Bossi *et al.*, 2009)
MA9133	Δ[*araBAD*]*76*::*rpoE* Tn*5*TPOP*45* (P^{BAD}-*rpoE*) *ompC6*::*lac* / pKD46	this work
MA9147	Δ[STM0869]::*cat*	this work
MA9242	Δ*yojN*::*cat*	this work
MA9346	*eptB115*::MudK Δ[STM0869]::*cat ΔrseA70*::*aadA*	this work
MA9380	Δ[*araBAD*]*76*::*rpoE* Tn*5*TPOP*45* (P^{BAD}-*rpoE*) Δ*ompD78*::*aadA ompC94::lac*	this work
MA9578	*ΔrseA70*::*aadA* Δ[STM0869]::*cat ompD96*::*lac*	this work

[a] All strains are derived from *Salmonella enterica* serovar Typhimurium strain MA3409. The latter is a strain LT2 derivative cured for the Gifsy-1 prophage (Figueroa-Bossi *et al.*, 1997).

Table 2. DNA oligonucleotides used in this work

name	Sequence (5'-3')[a]
pp813	GCGGAGGCTAGGGAGAGAGG
pp832	AGGGGTAAACAGACATTCAG
pp891	GCAGAGCTGGTACCAGGAGGGACAGTACTTTAACTTTCAT
pp893	*TTTTTTCATGCCTTATTCCGGCGTACAAATACGACGTTTT*GTGTAGGCTGGAGCTGCTTC
pp894	*AAGTCATTTTCATCGCTGTTTATCCTCATTCGAATGGACG*CCATGGTCCATATGAATATC
pp929	TGGGCTCCACAAAATGGGGACATCAAAGAAAAGCA
pp931	TATACCTCGGCTGCATTTACAACGCCTGCTGCCAACAG
pp952	GGTAATACGACTCACTATAGGGTTGCCGACTGGTTAATGAGGGTTA
pp953	GGAGGGACAGTACTTTAACTTTCATGTT
pp998	*TAAGGATTATTAAAATGAAACTTAAGTTAGTGGCAGTGGC*GATCCGTCGACCTGCAGTTC
ppA08	GTGCCGATCAACGTCTCATT
ppA11	*GTGACACGACATTGACACTCCGGCGATTCGCTTTACCATC*CCATGGTCCATATGAATATC
ppA12	*AAATAATTCAGGCCACACTGGAAGCGGTAAAGACCTATGG*GTGTAGGCTGGAGCTGCTTC
ppA40	CGCGCTAACCATCCTACCTT
ppA41	CCGCTCATTGAACCGATAGA
ppA62	TGACGGCGCTCTGTACCATA
ppA63	CGTTACCACGCTGCTGCATA
ppA68	GCCTTTGTCGTCAGAGAAGTA
ppA86	*GTACAGATCCAGTTTATTGCCGTCTTTGTTATATACCTCG*TGTGTAGGCTGGAGCTGCTT
ppB10	ACACTACCATCGGCGCTACG
ppB22	*AGAGGGTTAATAACATGAAAGTTAAAGTACTGTCCCTCCT*GATCCGTCGACCTGCAGTTC
ppB23	*AAACAGGTCTAATTTGTTGCCGTCTTTATTATAAATTTCA*TGTGTAGGCTGGAGCTGCTT
ppB31	*AATGAATTTCCAGACTCTTACGTCATGCCGGGCAACTTAACACG*CCTTACGCCCCGCCCT
ppB32	*CTAAAACTATCACGGTTATTCACTACACTCCCCTGCTCGAC*GCGCCTACCTGTGACGGAA
ppC46	ATGAAATTAATACGACTCACTATAGGCCACTGCTTTTCTTTGATGTCCCCATTTTGT
ppC47	ACAAAAAACCCACCAACCTTGAACCGAAAT
ppC57	ACAAAAAACCC**G**CCAACCTTGAACCGAAAT
ppD47	*AATTATAAGGATTATTAAAATGAAACTTAAGTTAGTGGCA***C***TGGC*GATCCGTCGACCTGCAGTTC
ppD48	*AATTATAAGGATTATTAAAATGAAACTTAAGTTA***C***TGGCAGTGGC*GATCCGTCGACCTGCAGTTC
ppD50	*TAGGTCGAACTTTTCGTTAAAGCATCAGTCATACCTATTGCCA***G***TGCTTTTCTTTGATGTC*
ppG24	*AATTATAAGGATTATTAAAATGAAACTTAAGTTA***C***TGGCA***C***TGGCGATCCGTCGACCTGCAGTTC*
ppG25	*AACTTAAGTTTCATTTTAATAATCCTTATAATTTTCTTAG*

[a]Changes generating mutations are shown in bold and underlined. Sequence extensions providing homology for recombination in gene swapping experiments are in italics.

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Chapter IV

Insertion hot spot for horizontally acquired DNA within a bidirectional small-RNA locus in *Salmonella enterica*

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Insertion Hot Spot for Horizontally Acquired DNA within a Bidirectional Small-RNA Locus in *Salmonella enterica*▽

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In *Escherichia coli* and *Salmonella enterica*, RyeA and RyeB RNAs are encoded on opposite DNA strands at the same locus. We present evidence indicating that the last 23 bp of the *ryeB* gene, corresponding to an internal portion of the *ryeA* gene, served repeatedly as the integration site for exogenous DNA during *Salmonella* evolution and still act as an attachment site for present-day bacteriophages. Interestingly, *ryeA* sequence and expression are modified upon lysogenization.

Lateral gene transfer is a prolific source of evolutionary changes in microorganisms and is thought to have had a major impact in the emergence of bacterial pathogens. In particular, the acquisition of so-called pathogenicity islands is regarded as being a key event in the conversion of ancestral extracellular bacteria into intracellular pathogens (2, 9, 11, 15). A common pathway to DNA acquisition involves the integrative recombination of circular DNA molecules into the host genome. The step is catalyzed by integrases, a class of site-specific recombinases encoded by temperate bacteriophages and plasmids. Integrases introduce staggered cuts at specific sequences on both donor and host DNAs and promote strand exchange and ligation (8, 14). As a result, the sequences recognized by the integrase are duplicated at each end of the inserted DNA. When conserved, such directed repeats allow a precise definition of the site of the original integration event.

Structure of the *Salmonella* CS 40 island. *Salmonella enterica* serovars harbor a panoply of genomic islands and islets. Some such elements are associated with prophage remnants, suggesting that they were incorporated as a result of lysogenization events. This is the case for a 16-kb insert lying at about centisome (CS) 40 on the chromosome map. The CS 40 island contains various loci linked to pathogenicity, such as *mig-3* (18) *pagK-pagO* (10), and *sopE2* (1, 16), interspersed with sequences reminiscent of phage genes. Among the latter is a presumptive integrase gene (STM1871) (12) located near one end of the island and oriented outwards. As in lambdoid prophage maps (*int* gene near *attL*), the STM1871-proximal end is hereafter designated the "left" end of the island (Fig. 1). Alignment with the *Escherichia coli* K-12 genome sequence reveals that the *Salmonella* CS 40 island is inserted into the intergenic region between the *pprA* gene (also named *pphA*) and the ortholog of the *yebY* locus (STM1873) (Fig. 1). Recently, the *prpA-yebY* intergenic region of *E. coli* was shown to contain two small-RNA (sRNA) genes with opposite polarities, *ryeA* and *ryeB*, transcribed from opposite DNA strands, with the *ryeB* sequence entirely contained within the larger *ryeA* gene (19, 20). The core region of this locus, a 146-bp segment comprising the entirety of *ryeB*, is highly conserved in *Salmonella* and is located immediately to the left of the CS 40 island near the end of the putative integrase gene.

Since we wondered if the interval between *ryeB* and STM1871 contained the integrase recognition site, we examined whether a portion of the sequence was repeated on the opposite end of the island. No such repeat could be identified at the right end of the element; however, a sequence identical to the last 23 bp of the *ryeB* gene, and in the same orientation, was located inside the island, approximately 10 kb from the left end. Interestingly, this sequence lies adjacent to an open reading frame (STM1861) whose putative product shares 77% identity (89% similarity) with the C-terminal half of the STM1871-encoded integrase. This strongly suggests that the CS 40 island is in fact made of two separate islets lying side by side, one carrying *mig-3* and *pagKO* (left) and the other containing the *sopE2* gene (right) (Fig. 1). The lack of a recognizable attachment site at the right end of the insert, as well as the apparent defective nature of STM1861, suggests that the *sopE2* islet was acquired earlier and has since suffered extensive decay. Consistent with this idea, *Salmonella bongori*, a lineage sharing a common ancestor with *Salmonella enterica*, carries *sopE2* (13) but lacks the *mig-3–pagKO* islet (data not shown).

The above-described findings tentatively define the last 23 bp of the *ryeB* gene as the core region of the integration site. Since this sequence is reconstituted upon integration, the acquisition of the CS 40 islets is not expected to have affected *ryeB* gene structure or expression. In contrast, if the *ryeA* gene is positioned in *Salmonella* as in *E. coli*, the incorporation of the islets should have separated the structural portion of the gene from its original promoter. Thus, it seemed relevant to determine the *ryeA* transcriptional status in *Salmonella*. For this purpose, RNA extracted from *S. enterica* serovar Typhimurium strain LT2 was subjected to Northern hybridization analysis using oligonucleotides complementary to the predicted sequences of RyeA and RyeB RNAs as probes. As shown in Fig. 2, both probes gave positive signals. In the case of RyeA, the most intense band corresponded to an RNA of 250 to 300 nucleotides, while the RyeB analysis detected an RNA of approximately 100 nucleotides. These sizes are closely comparable to those of RyeA and RyeB RNAs in *E. coli* (19,

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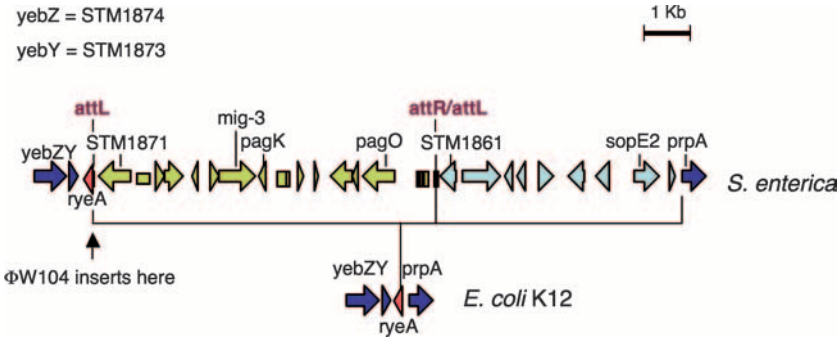

FIG. 1. Organization of the CS 40 island in *Salmonella enterica* serovar Typhimurium and of the corresponding region in the *E. coli* chromosome. Horizontal arrows represent open reading frames. The arrow clusters depicted in green and light blue correspond to segments proposed to originate from separate insertion events (see text). Phage ΦW104 inserts at the left boundary of the island. The diagram is based on data from references 10, 12, 16, 19, and 20).

20). As observed in *E. coli* (20), additional fainter signals were detected with both probes. These minor bands are likely to represent processing products.

A survey of the *Salmonella* genome sequences revealed the occurrence of yet another insertion event at the *ryeA*/*ryeB* locus. Some strains carry a prophage-related insert between the end of *ryeB* and the left boundary of the *mig-3–pagKO* islet (Fig. 1). The length and structure of the *Salmonella ryeA*/*ryeB* locus insert vary considerably, from a full-length prophage in some isolates (e.g., *S. enterica* serovar Typhi strain CT18) (17) to a shortened and scrambled version in others (e.g., *S. enterica* serovar Enteritidis strain LK5) (7). In all instances, the terminal 23 to 26 bp of *ryeB* are found duplicated at the two ends of the element, indicating that this portion of the gene serves as the attachment site. Thus, in strains carrying the *ryeB*-linked prophage, *ryeA* transcription is expected to originate from within phage DNA.

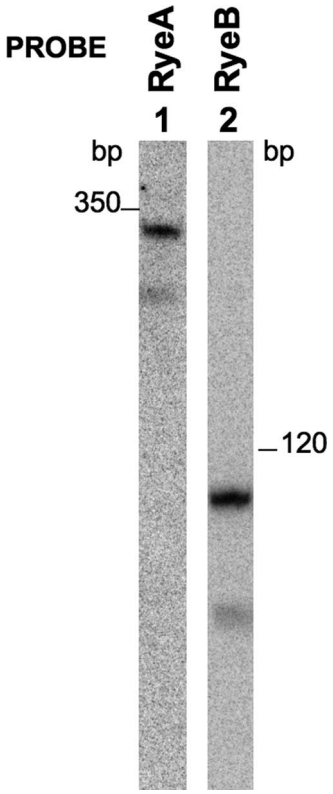

FIG. 2. Northern blot analysis of RyeA and RyeB RNAs in *Salmonella enterica* serovar Typhimurium LT2. Cultures grown overnight in LB were diluted 1:200 in fresh LB and grown to an optical density at 600 nm of 0.35. RNA was extracted as previously described (4), fractionated on a 6% polyacrylamide–8 M urea gel (lane 1) or on an 8% polyacrylamide–8 M urea gel (lane 2), transferred onto a Hybond-N^+ membrane, and hybridized to ^{32}P-labeled oligonucleotides complementary to RyeA (pp925 [5′-GGAAAACCTGGCGTCGTCATCTATTCTTAAAGGGCAAGGCGA-3′]) and RyeB (pB13 [5′-GATTCCTGTATTCGGTCCAGGGAAATGGCTCTTGGGAGAGAG-3′]). Sizes were estimated from migration distances of tmRNA and 5S RNA (not shown).

While tRNA and tmRNA genes constitute a favored target for temperate phages and other integrative elements (21), to our knowledge, only one example of the phage insert in an sRNA gene has been reported. Wassarman and colleagues found the previously mapped *attB* site for bacteriophage P2 in *E. coli* to correspond with the 3′ end of the *ryeE* gene, which encodes an Hfq-binding sRNA of unknown function (20). In both the *ryeB* and *ryeE* genes, the attachment site lies within the sequence encoding the stem-loop of the transcription terminator, suggesting that the region of dyad symmetry participates in integrase recognition (14, 21).

Effect of phage integration on *ryeA*/*ryeB* expression. As part of a separate study, we examined the occupancy of the *ryeA*/*ryeB att* site in 84 *Salmonella enterica* serovar Typhimurium isolates of human or animal origin using a three-primer-based PCR assay. This analysis revealed the presence of a DNA insert in a fraction of the strains. Interestingly, all positive strains belonged to the DT104 phage type, suggesting that the acquisition of the insert occurred recently, possibly coinciding with the emergence of the virulent epidemic clone (3; N. Figueroa-Bossi, F. X. Weill, P. A. Grimont, and L. Bossi, unpublished data). Sequence data from the Sanger Institute website (http://www.sanger.ac.uk/Projects/Salmonella/) show the *ryeA*/*ryeB*-associated element to be a full-size lambdoid prophage. To assess its functional state, we deleted the prophage from the genome of a DT104 strain in our collection, MA6711 (5), and used the resulting strain (MA7860) as a host for monitoring the release of plaque-forming particles in cultures of the MA6711 parent. Tiny plaques from which active

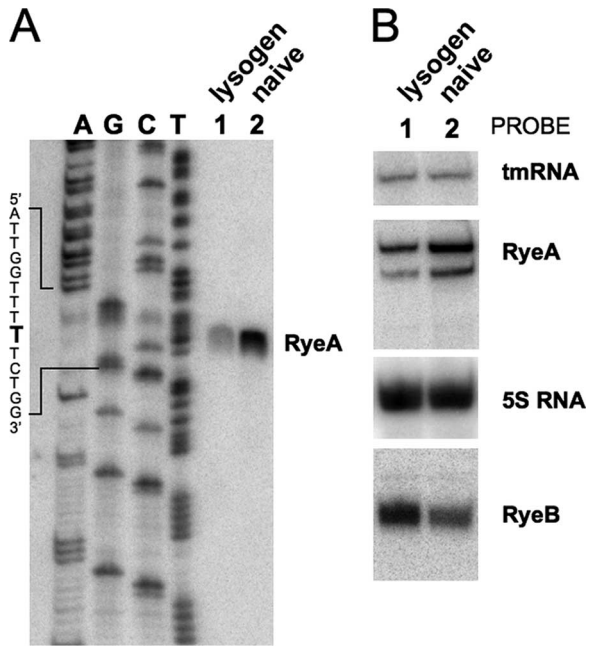

FIG. 3. Effect of phage ΦW104 integration on *ryeA*/*ryeB* expression. Bulk RNA was extracted from strains LT2 and MA7833 (ΦW104 lysogen) as described in the legend to Fig. 2. (A) Primer extension analysis of RyeA RNA from the lysogenic strain (lane 1) and from wild-type LT2 (lane 2). Reverse transcriptase reactions were carried out using primer ppB12 (5′-CCCTGGACCGAATACAGGA-3′) as previously described (6). Sequencing reactions were performed with the *fmol* DNA cycle sequencing system from Promega according to the manufacturer's protocol. The template was a DNA fragment obtained by PCR amplification of chromosomal DNA from strain MA7833 with oligonucleotides pp490 (5′-TGGCGTCGTCATCTATTC-3′) and pp491 (5′-CAGGGACGCTATCACACA-3′) as primers. (B) Northern blot quantification of RyeA and RyeB levels in strains MA7833 (lane 1) and wild-type LT2 (lane 2). Bulk RNA was fractionated on an 8% polyacrylamide–8 M urea gel. Membranes were probed with ^{32}P-labeled oligonucleotides pp925 and ppB13 (see the legend to Fig. 2). 5S RNA and tmRNA probed with ppB10 (5′-ACACTACCATCGGC GCTACG-3′) and pp813 (5′-GCGGAGGCTAGGGAGAGAGG-3′), respectively, were used as internal controls. Due to the higher gel concentration, the two RyeA bands are less separated than in Fig. 2.

virus could be isolated and characterized were detected. The phage, hereafter named ΦW104, proved capable of infecting and lysogenizing a variety of serovar Typhimurium strains including LT2, ATCC 14028, and SL1344. The isolation of such lysogenic derivatives provided a system for studying how ΦW104 integration affected the expression of the *ryeA*/*ryeB* locus. To identify the *ryeA* promoter, RNA preparations from exponential cultures of strain LT2 and its ΦW104-lysogenized derivative MA7833 were subjected to primer extension analysis (Fig. 3A). These experiments located the 5′ end of RyeA RNA at identical positions in the two strains, approximately 80 bp to the right of the *ryeB* gene (Fig. 4). The presence of sequences resembling the −10 and −35 consensus motifs of σ^{70}-dependent promoters immediately upstream from the 5′-end position is consistent with this being the *ryeA* transcription start site. Overall, the sequences around this region in naive and lysogenic strains are highly conserved (Fig. 4), with the identity extending to the ΦW104 putative *int* gene that strongly resembles STM1871 (see above). Nonetheless, the difference in the intensities of the primer extension bands shown in Fig. 3A suggested that RyeA RNA might be more abundant in strain LT2 than in its lysogenic derivative. The difference was confirmed by Northern blot hybridization analysis (Fig. 3B). Interestingly, RyeB followed an opposite trend, being synthesized at a lower level in LT2 than in MA7833 (Fig. 3B). Since the *ryeB* sequence is unaffected by the lysogenization event, the observed difference might reflect the change in *ryeA* transcription associated with such an event. Conceivably, RNA polymerases transcribing the *ryeA* gene could dislodge polymerases bound to the *ryeB* promoter, causing the activity of the latter to negatively correlate with that of the *ryeA* promoter. Some variability in the sequence upstream from the *ryeA* promoter, particularly a 9-bp deletion/insertion at position −49 (Fig. 4), might account for the difference in *ryeA* transcription rates.

Biological significance of prophage insertion at the *ryeA*/*ryeB* locus. The above-described data suggest that the integration of the ΦW104 prophage "resets" the levels of RyeA and RyeB RNAs in the cell. In addition, the 5′ portion of RyeA RNA is changed upon lysogenization. It is temping to specu-

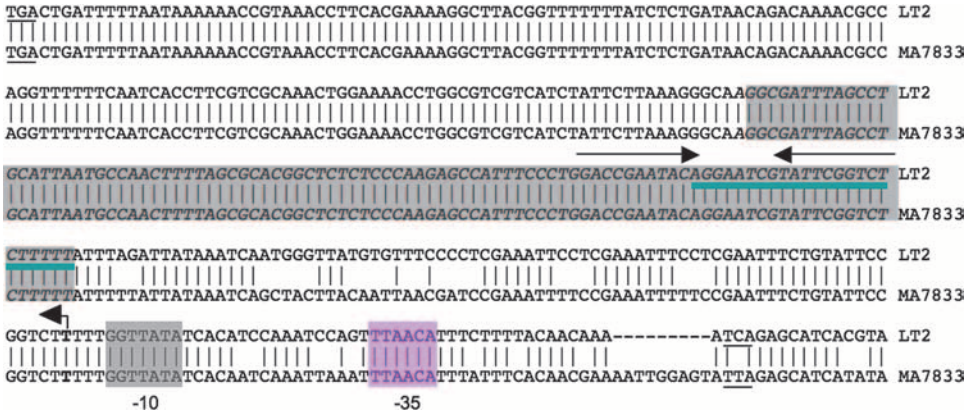

FIG. 4. Sequence alignment of *ryeA*/*ryeB* chromosomal regions in LT2 and ΦW104 lysogen. Underlined triplets indicate the translation termination codon of *yebY* and the complements of termination codons of STM1871 (LT2) (12) and the ΦW104 *int* gene (MA7833). The sequence of the *ryeB* gene is in italics and shaded. Gene boundaries are inferred from the 96% identical *E. coli* sequence (19, 20). Facing arrows indicate the transcription terminator stem sequence. Green underlining indicates the sequence found duplicated at the two ends of the ΦW104 prophage. The left-pointing arrow defines the *ryeA* transcription start site. Purple boxes indicate *ryeA* promoter elements.

late that these modifications might have physiological consequences. Unfortunately, the lack of information on the physiological roles of RyeA and RyeB does not offer much grounds for such speculation. RyeB RNA was shown to strongly bind the Hfq protein (20), suggesting its involvement in some Hfq-mediated regulatory mechanism. In contrast, RyeA bound Hfq with low affinity (20). Possibly, the role of this RNA is limited to regulating RyeB levels through transcriptional interference, as suggested above, or by a direct RNA-RNA interaction (19).

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Discussion

"Ciencia es todo aquello sobre lo cual siempre cabe discusión."

José Ortega y Gasset

At the beginning of the studies described in this thesis, the predominant view regarded with skepticism the possibility of applying classical genetic approaches in the study of sRNAs (reviewed in [323]). The main reason for such thinking was that no sRNA had ever been discovered by mutation. This was ascribed to the small size of the sRNA genes (unlikely targets for mutagenesis) combined to the absence of clear-cut phenotypes associated with defects in sRNA functions (reviewed in [323]). Because of this, the vast majority of studies on sRNAs were carried out by transcriptomic analyses using strains overproducing sRNAs from multi-copy plasmids. There is no doubt that this approach has been critical for discovery of sRNAs and their mRNA targets, as well as for the elucidation of the general mechanism of sRNAs function (reviewed in [249, 259, 263, 323]). However, sRNA overexpression analyses do not faithfully reproduce the physiology of the mechanism being studied and are potentially susceptible to artifacts due to gene dosage effects.

These considerations prompted us to develop genetic systems that would allow us to study sRNA-mediated regulation under conditions where the sRNA genes and their targets are present in single-copy in their natural chromosomal context (chapters 2 and 3) [4, 52, 118]. This genetic approach has proven extraordinarily useful in detecting sRNA-regulated genes (chapter 2) and identifying sRNAs affecting a given target [52]. Mutational analysis allowed gathering insight on structural and functional features of sRNA-mediated regulatory mechanisms (chapter 3) [4, 52, 118]. Finally, the same approach led to the discovery of a novel mechanism through which the regulatory activity of an sRNA can be itself regulated by an RNA mimicking the target of that sRNA [52].

Constitutive induction of σ^E-dependent envelope stress response in *hfq* mutants

Studies presented in chapters 1 and 2 of this thesis aimed at identifying potential targets of regulatory RNAs in *Salmonella*. Genes whose expression varied in the presence or absence of Hfq were identified by transcriptomic

analysis and by a genetic approach using randomly inserted *lacZ* translational fusions.

Genetic and transcriptomic analyses allowed establishing a list of genes regulated, either positively or negatively, by Hfq. Expression of these genes could be either controlled by the Hfq protein directly or regulated by an sRNA with participation of the chaperon protein. Genes in the list encode proteins involved in essential physiological functions, such as nutrients uptake and metabolism, gene regulation, stress resistance, DNA repair, conjugal transfer, motility, biofilm formation and expression of virulence factors (chapters 1 and 2). These data provided insight on the relevance of Hfq protein in cellular functions and provide a list of candidates genes putatively regulated by sRNAs, some of them were subsequently subject of further investigation (chapters 2, 3 and 4) [4, 52, 118].

Besides putative sRNA-controlled genes, results revealed others genes for which the effect observed in a strain lacking Hfq reflected defective or constitutive activation of σ^S and σ^E responses, respectively. In the case of RpoS, these results were not surprising since full RpoS expression is dependent on sRNAs (and therefore on Hfq) [112-116]. A link between Hfq and σ^E was previously reported in *Vibrio cholerae*, although the basis for this relationship was not addressed [324].

The study described in chapter 2 revealed that induction of the σ^E regulon in a *Δhfq* strain results from activation of RseA cleavage by DegS protease. The relatively high number of genes encoding envelope proteins found upregulated in the *Δhfq* strain suggested that σ^E activation could be linked to accumulation of one or more misfolded or unfolded proteins in the periplasmic space (chapter 2).

In the course of this study, it became apparent that the constitutive activation of σ^E, due to the removal anti-σ^E factor RseA causes an abrupt inhibition of the synthesis all major porins, which correlates with the disappearance of *omp* mRNAs in Northern blots. Interestingly, no such inhibition was observed in a Δ*hfq* background, despite the levels of σ^E

activation in this strain approach those of an *rseA* mutant. This suggested that the factors responsible for porin inhibition were σ^E-dependent sRNAs, which could not function in the Δ*hfq* background (chapter 2, Figure 5). Indeed, a number of independent studies subsequently showed that OMP repression under σ^E inducing conditions is largely due to the action of two sRNAs, MicA and RybB sRNAs [1, 3, 4, 59, 74] (reviewed in [72, 81-83]). Given that Hfq protein is necessary for this sRNA-mediated regulation, MicA and RybB will no longer repress OMP expression in absence of Hfq, leading to increased production of OMPs. Overproduction of porins or other OMPs could cause some of these proteins to fold improperly and accumulate in the periplasm, leading to DegS activation [154, 325].

Indeed, a further study showed various OMPs (particularly OmpD, LamB, and OmpC) to collectively contribute to the induction of σ^E response in the *hfq* mutant [118]. Intriguingly, the deletion of both *micA* and *rybB* genes activated σ^E to a much lesser extent than the *hfq* deletion, suggesting that loss of MicA and RybB does not solely account for the observed σ^E activation [118]. Perhaps Hfq represses OMPs directly, through an sRNA-independent mechanism. Alternatively, OMP repression could involve the participation of additional yet unidentified sRNAs.

Activation of σ^E (and subsequent downregulation of *omp* genes) is not restricted to stress conditions; rather, it is an integral part of the control system of OMP homeostasis during normal growth and in the transition between exponential and stationary phases. MicA and RybB sRNAs play a role in this regulation [326], as illustrated by the model in figure D1.

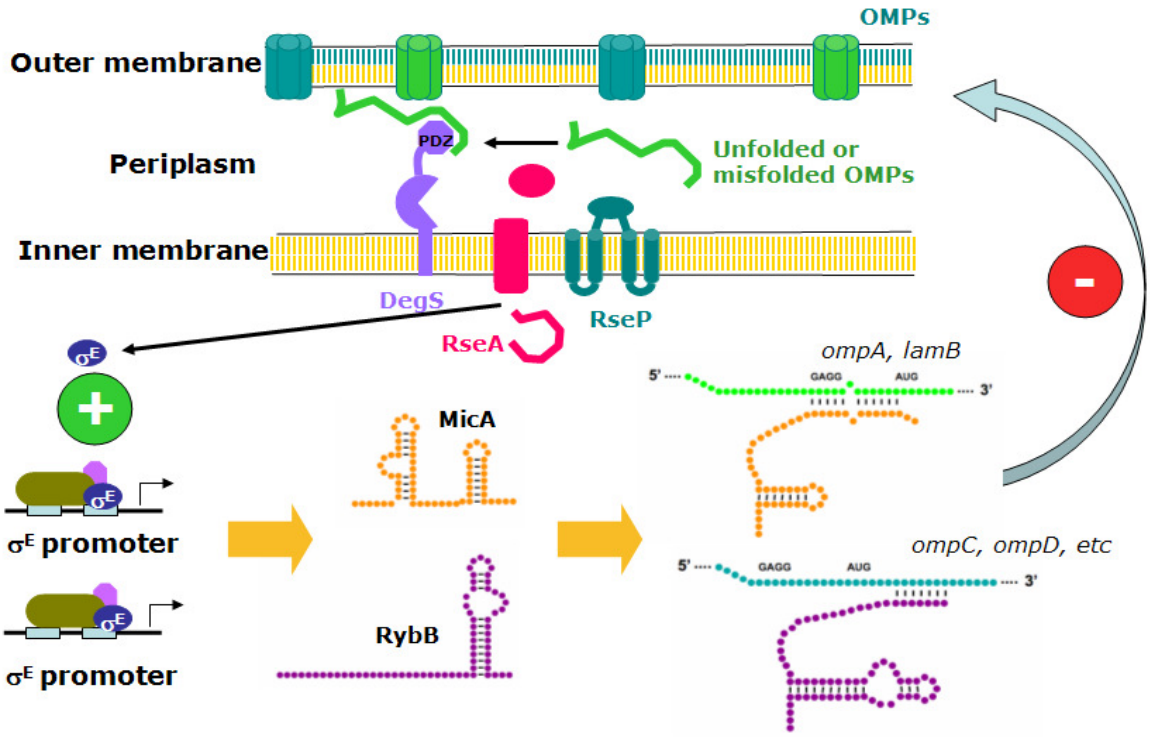

Figure D1. Envelope homeostasis. Under non-stress conditions, σ^E is inactive because it is sequestered by its specific anti-sigma factor RseA. Stress causes the appearance of misfolded OMPs in the periplasm, which elicit the release of σ^E to the cytoplasm. Free σ^E can associate with RNA polymerase to activate transcription of MicA and RybB. The σ^E-dependent synthesis of MicA and RybB leads to feedback inhibition of OMP accumulation, the main cause of σ^E activation.

The work presented in chapter 2 constituted the first demonstration of an Hfq involvement in the regulation of bacterial envelope biogenesis. After this work was published, similar results were reported for *E. coli* [297]. Altogether these data suggests that sRNA function is not restricted to stress conditions [293], and it might be integral part of the normal physiological control of gene expression as a function of growth conditions (see also [52]).

Dissecting the functional anatomy of RybB sRNA

The two σ^E–dependent sRNAs, MicA and RybB, inhibit translation of several OMPs in an Hfq-dependent manner. MicA downregulates OmpA [59], LamB [4] and also the two-component system proteins PhoPQ [327], whereas RybB has been shown to repress OmpN, OmpC, OmpD, OmpW, OmpF, OmpA, OmpS, FadL and ChiP (chapter 3) [3, 74].

Sequence elements important for the function of MicA sRNA were identified through the analysis of mutations affecting the MicA-dependent regulation of a *lamB-lacZ* fusion [4]. A similar genetic approach was used to characterize the functional domains of RybB sRNA (chapter 3). Mutations affecting the ability of RybB to downregulate an *ompC-lacZ* fusion were selected following mutagenic PCR. Mutations could be separated in two groups: those affecting *rybB* gene promoter region (not further investigated) and mutations in the structural part of *rybB*. This second class included mutations in three separate regions of the sRNA molecule: the 5' end, the center and the 3' end (chapter 3).

RybB pairing domain

Mutations at the 5' end of RybB mapped within the sequence previously shown to base pair with *ompN* mRNA [1]. The same RybB sequence pairs with *ompC* mRNA (chapter 3, Figure 1A), suggesting that 5' end of RybB might be involved in the regulation of other known targets [35], as has been recently demonstrated for OmrA/OmrB sRNAs [71]. However, while RybB:*ompN* pairing takes place within the coding sequence, the region of *ompC* mRNA that pairs with RybB is located in the 5'-UTR, 40 nucleotides upstream from the start codon (chapter 3, Figure 1A), and also upstream from the interaction site of another sRNA acting on *ompC* mRNA, namely, MicC [68]. Given that the RybB:*ompC* pairing site lies outside the -35 +19 region covered by the 30S subunit of the ribosomes [328], it seems difficult to invoke an obstruction to ribosomal access as the cause of *ompC* repression. Nevertheless, inhibition of mRNA translation initiation by sRNA pairing upstream of a 30S-binding site has been reported [61]. An alternative mechanism for RybB action on *ompC,* in which formation of the duplex might stimulate cleavage by ribonucleases, cannot be discarded [41, 56]. Further experiments will be needed to distinguish between these two possibilities.

Our *in vitro* results show that Hfq binds the region of *ompC* between RybB pairing segment and the start codon, consistent with the presence of two putative high-affinity binding sites (AAUAA) within the region. The upstream

motif is within the sequence recognized by MicC sRNA [68]. This raises the possibility that MicC pairing cannot take place at the same time as RybB pairing since the latter presumably occurs with Hfq bound at the MicC-pairing site. The downstream Hfq-binding motif on *ompC* mRNA lies between the Shine-Dalgarno (SD) sequence and the start codon. This site might be required for MicC function. Alternatively, Hfq might act on *ompC* directly, blocking ribosome access or promoting *ompC* mRNA destabilization [56]. The latter possibility is in agreement with previous data showing that the effect of *hfq* mutation on OMP repression are partially independent of MicA and RybB sRNAs [118].

The study of *ompD* regulation by RybB revealed the presence of two pairing sequences for RybB sRNA within the initial portion of *ompD* mRNA (chapter 3, Figure 3A). Both interaction sites are functional *in vivo* although, because of their proximity, they are mutually exclusive. Hence, the duplication does not increase the efficacy of RybB repression. In fact, the opposite may be true as the two sites appear to interfere with each other (chapter 3, Figure 3B). Perhaps a sterical impairment might occur between two RybB molecules simultaneously trying to access the two positions (chapter 3).

Hfq binding domain

Mutations mapping in the center of the sRNA molecule showed to cause a decrease in RybB levels (chapter 3, Figure 4), suggesting that they might affect stability of the sRNA rather than its activity. Mobility shift assays demonstrated that alleles U30C and G33A decreased the affinity of RybB for Hfq (chapter 3, Figure 5). The destabilization might therefore be a consequence of a less efficient Hfq binding. In contrast, the effects of the C37U allele were ascribed to a destabilization of the transcription termination hairpin, causing increased sRNA turnover.

Terminator domain

The sequence at the 3’ end of RybB shows a GC-rich stem loop structure followed by a run of U residues characteristic of Rho-independent

transcription terminators. *rybB* mutations clustered in the 3' arm of the stem cause partial or total destabilization of the whole molecule (chapter 3, Figure 4). This supports the idea that terminator structure is a stabilizing element in bacterial RNAs [51, 329, 330]. One of the mutations on the stem (U70C allele) is able to form an alternative structure by slippage, shortening the stem loop and extending the single-stranded region (chapter 3, Figure 7). This structural change does not affect RybB stability or Hfq binding, but nonetheless impairs RybB activity. Whether this defect is a mere consequence of the altered shape of the sRNA molecule, or reflects a direct participation of the terminator step in RybB function (e.g. contribution to pairing), remains to be determined.

A new member of the RybB regulon

ChiP chitoporin is an additional target of RybB-mediated downregulation. Synthesis of ChiP depends on the presence of chitosugars in the medium [52]. The induction mechanism involves inactivation of ChiX sRNA (MicM), which normally inhibits translation of *chiP* mRNA [52, 76, 78]. Upon induction, ChiP is highly expressed [52]. Thus, it is not surprising that under these conditions, *chiP* may also be subject to regulation by one of the major sRNA regulators involved in membrane homeostasis.

Heptameric "seed" sequence

The 5' end of the RybB molecule is the main determinant of recognition of *ompN* [1], *ompC*, *ompD* and *chiP* target mRNAs (chapter 3). Specifically, the first 7 bases of the RybB sequence play a predominant role in the interaction with target mRNAs. This situation resembles postranscriptional regulation by eukaryotic microRNAs (miRNAs), where the minimal requirement for regulation consists of a 7-base "seed" sequence [331, 332]. Thus, it could be a general feature inherent to RNA antisense regulation.

Examination of the sequences of other targets [3, 74] showed additional putative pairing sites for the RybB "seed" sequence in *ompW*, *ompA*, *ompF* and *ompS* mRNAs. In *ompF* mRNA, a putative RybB pairing sequence is

located approximately 40 nucleotides upstream from the start codon, and also upstream from the interaction sequence for MicF sRNA [5, 6] (Figure D2). This scenario is reminiscent of the situation of RybB and MicC interaction sequences in *ompC* mRNA (chapter 3, Figure 1A) [68], suggesting the involvement of similar mechanism in both cases.

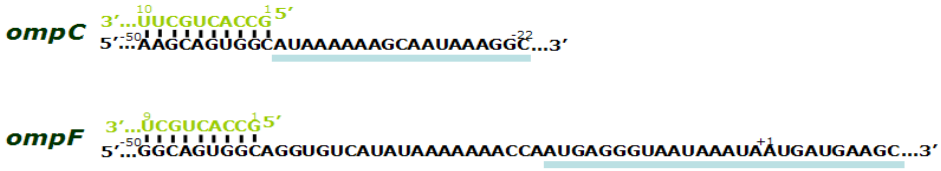

Figure D2. RybB-pairing sequence in *ompC* (chapter 3) and predicted model of interaction with *ompF* mRNAs. RybB sequences are highlighted in red, whereas mRNA sequences are represented in black. Blue bar indicates MicC [68] and MicF [5, 6] pairing sequences.

Strikingly, all three *ompW*, *ompA* and *ompS* mRNAs present two putative RybB pairing sites within their coding sequence, as in the case of *ompD* (chapter 3) (Figure D3). Thus, the "double pairing" pattern appears to be a widespread feature of RybB-target interaction. However, its role in regulation, if any, remains elusive.

In summary, RybB pairing with target mRNA could take place either at the 5'-UTR region, far upstream from the start codon (*ompC*, *ompF*), or within the coding sequence (*ompN* [1], *ompD*, *chiP*, *ompW*, *ompA* and *ompS*). In the latter case, RybB can pair with either one (*ompN* [1] and *chiP*) or two mRNA sites (*ompD*, *ompW*, *ompA* and *ompS*) (Figure D2).

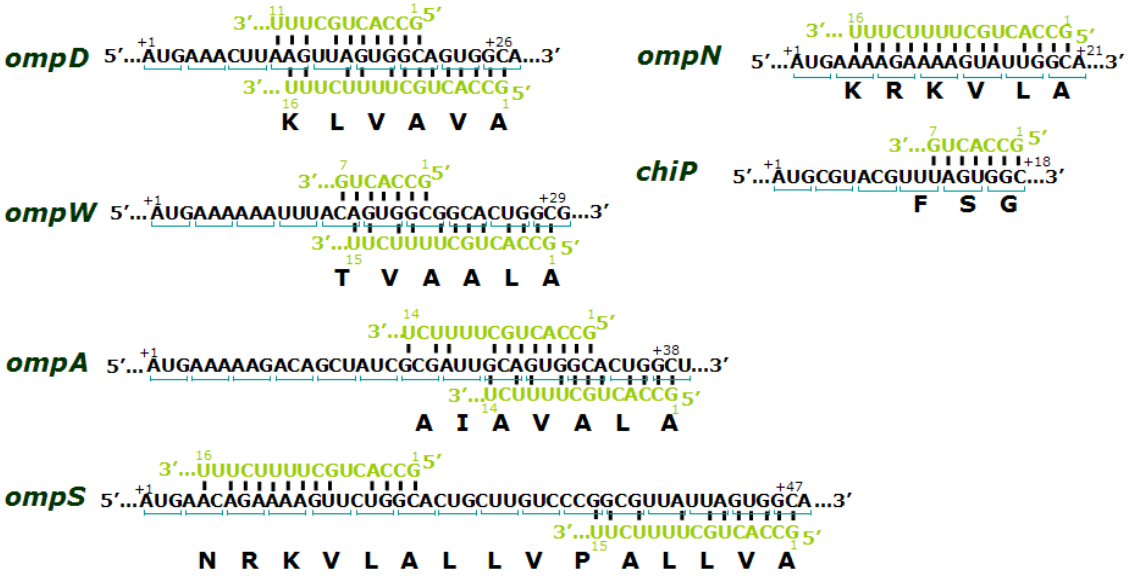

Figure D3. RybB-pairing sequences in *ompD* (chapter 3) and *ompN* [1] mRNAs and predicted models of interactions with *ompW*, *ompA* and *ompS*. RybB sequence(s) is (are) highlighted in red, whereas mRNA sequences are represented in black. Codons are underlined in blue and amino acid sequences involved in pairing are marked.

Interestingly, the RybB-pairing sequences in *ompN* [1], *ompD*, *chiP*, *ompW*, *ompA* and *ompS* lie to the signal peptide encoding regions of these mRNAs. In addition, pairing sequences in all mRNA but *chiP* are in the same frame (Figure D3). This suggests the possibility that RybB might have adapted to recognize sequences that are conserved because of the function of their peptide products. However, finding that the RybB pairing sequence in *chiP* mRNA is in a different reading frame argues against this interpretation and supports the quite opposite view: namely that the compatibility of the seed sequence with signal peptide function in different reading frames is a key feature driving the evolution of RybB target specificity.

sRNAs and horizontal transfer

Salmonella enterica harbors a plethora of genomic islands and islets of horizontally acquired DNA. Integrative recombination is commonly involved in such DNA acquisition. This reaction is catalyzed by a class of site-specific recombinases called integrases, encoded by temperate phages or plasmids. Integrases produce a staggered cut at specific sequences on both donor and host DNA, promoting strand exchange and ligation, resulting in a duplication of the sequence initially recognized [239, 240]. Thus, the integration event generates short direct repeats on the two sides of the integrated DNA. Such

direct repeats are often conserved, allowing one to accurately define the site where the insertion event occurred.

Hotspot for integration events

RyeA and RyeB sRNAs are encoded on opposite strands of the same locus in *E. coli* and *Salmonella* The *ryeA* gene includes the entire sequence of *ryeB* [29, 31, 32]. In *Salmonella*, the region downstream from the *ryeB* gene (*yebY-prpA* intergenic region, around centisome 40 in the chromosome) contains an additional 16Kb insert (CS 40 island) encoding several genes whose products are linked to pathogenesis. These virulence-related genes, as *mig-3* [333], *pagK-pagO* [334] and *sopE2* [335, 336], are interspersed with sequences reminiscent of phage genes, among which there is a putative integrase (*STM1871*) [13] located on the "left" end of the island (chapter 4, Figure 1). This suggests that an additional integration event occurred in *Salmonella*, involving these 16Kb not present in *E.coli*. The core region of the *ryeAB* locus (a 146 bp region including the complete *ryeB* gene) is highly conserved in *Salmonella* and is located immediately to the "left" of the island, close to the end of the putative integrase gene. Since the region between *ryeB* and *STM1871* might contain the integrase recognition site, we examined whether the sequence was present on the opposite end of the 16Kb island (close to *prpA* gene). No such repeated element could be identified at the "right" end of the island. However, a sequence identical to the last 23bp of *ryeB*, and in the same orientation, was detected within the island. Interestingly, this sequence lied adjacent to a gene (*STM1861*) whose product is similar to the c-terminal end of *STM1871*-encoded integrase. This strongly suggests that the CS 40 island is in fact formed of two different islets located side by side, and resulting from independent integration events. The left islet harbors *mig-3* and *pagKO* while the right islet carries the *sopE2* gene (right) (chapter 4, Figure 1). Since there is no recognizable attachment site at the right end of the insert, and because of the apparent defective nature of *STM1861*, it seems that the *sopE2* islet was acquired earlier, undergoing extensive decay since.

Interestingly, the property of the ryeA/ryeB locus to serve as integration site for laterally acquired DNA in *E. coli* had been independently predicted by an *in silico* study [247].

Effect of the insertion on gene expression

The last 23bp of the *ryeB* gene appear to constitute the core region of the integration site. Given that this sequence is reconstituted upon integration, acquisition of CS 40 is not expected to have affected *ryeB* structure or expression. In contrast, *ryeA* expression might have been affected since the insertion lies between the promoter and the structural portion of the gene. To a first sight, however, the expression pattern of RyeA and RyeB in *Salmonella* appear similar to those of *E. coli* (chapter 4, Figure 2) [29, 31, 32].

Some *Salmonella* strains carry an additional (third) insertion between the end of *ryeB* gene and the left boundary of the *mig-3-pagKO* islet (chapter 4, Figure 1). This prophage-related insert largely varies in length and structure in different *Salmonella enterica* serovars (chapter 4) [337, 338]. In all cases, the terminal 23-26bp of *ryeB* appeared duplicated at the two ends of the element, indicating that this sequence also served as the attachment site for this integration. Thus, in strains carrying the insertion, *ryeA* transcription is expected to originate from within phage DNA. Because of this, transcription rate and/or *ryeA* structure might be modified upon prophage integration. Comparison of RyeA 5′ ends in *Salmonella* Typhimurium LT2 and a lysogenic derivative carrying the insertion showed that there are no changes in the positions of the transcription start sites. Consistent with this, sequences in naive and lysogenic strains are conserved over this region, and contain boxes resembling to -35 and -10 consensus motifs of σ^{70}–dependent promoters upstream from RyeA 5′ end (chapter 4, Figure 4).

However, Northern analysis showed lower amounts of RyeA RNA in the lysogenic strain compared to the naive strain. In contrast, RyeB levels were higher in lysogenic strain. This inverse relationship in RyeA/RyeB levels had

been previously reported [31, 32]. A mechanism based on base pairing between both sRNAs, resulting in a duplex-dependent Rnase III processing, has been proposed to explain this observation [31]. Given that *ryeB* is unaffected by the insertion, the observed difference in RNA levels must stem from a change in *ryeA* transcription subsequent to this insertion event. Variations in transcription rates can might result from differences in the region upstream from its promoter. Altogether, this data suggest that integration alters the relative levels of RyeA and RyeB RNAs in the cell, besides changing the 5' portion of RyeA. The lack of information on the physiological roles of RyeA and RyeB sRNAs does not allow further speculation on the biological consequences of these changes.

sRNAs and evolution

To summarize, the *ryeAB* locus appears to serve as insertion hotspot for horizontally acquired DNA (chapter 4). In *E.coli*, *Shigella* and *Salmonella* there have been different integration events at that locus (chapter 4) [247], some of them including genes whose product contribute to pathogenesis (chapter 4) [333-336]. A recent phage insertion in that locus "resets" RyeA and RyeB levels, slightly altering RyeA 5' sequence as well (chapter 4).

Other sRNA genes have been reported to be associated with genomic islands [32, 88, 237, 238], indicating that, as it is the case of tRNAs and tmRNA [241-244] (reviewed in [245, 246]), sRNAs can act as preferred integration sites for horizontally acquired DNA. These include the gene of RybB, the subject of chapter 3 of this thesis, which was reported to be the site of insertion of a genomic island in *E.coli* strain CFT073 [247]. It has been proposed that the archetypal symmetric stem-loop structure of the Rho-independent transcriptional terminator of the sRNAs could play a role in integrase recognition [240, 244] (reviewed in [246]).

Occasionally, aberrant excision of the integrative element leads to the incorporation of neighboring host DNA in the excised fragment [248]. In light of the frequency of sRNA genes at insertion sites, it is not difficult to imagine that movable elements may "pick up" sRNA genes. This could

account for the presence of such genes in genomic islands. Island-encoded sRNAs are able to regulate expression of genes belonging to the core genome, and participate in essential processes as membrane remodeling and virulence [88, 238]. Finally, integration of insertion elements can also affect sRNAs expression (chapter 4).

In conclusion, some sRNAs constitute records of integration episodes (chapter 4), disseminate with movable elements [88, 237, 238, 247], affect core genome gene expression patterns [88, 238] and can be altered by further insertion events (chapter 4). All of these properties and functions make sRNAs key players in bacterial evolution.

Conclusions

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That’s funny...'.”

Isaac Asimov

1- Two independent general approaches, namely, the genetic and transcriptomic strategies, allowed us to identify genes regulated by the RNA chaperon protein Hfq. These include putative sRNA-regulated genes and others controlled, either directly or not, by Hfq.

2- The list of genes identified provides insights into the key role of Hfq and sRNAs in the regulatory control of essential cellular processes, such as nutrients uptake, metabolism, gene regulation, DNA repair, stress resistance, conjugal transfer, motility, biofilm formation and expression of virulence factors. A set of Hfq regulated genes was found to be part of σ^S regulon, confirming the role of Hfq in sRNA-mediated activation of *rpoS*.

3- Some genes downregulated by Hfq were found to be σ^E-dependent. This finding revealed constitutive activation of σ^E response in an Hfq^- background. Loss of Hfq cause an increase on cleavage of RseA anti-sigma factor. Negative control of OMPs biogenesis by sRNAs and Hfq suggest involvement of OMPs overproduction in constitutive activation of σ^E response in *hfq* mutants. First demonstration that MicA is a σ^E-controlled sRNA. The antagonistic relationship between σ^E-dependent sRNAs and σ^E response activation demonstrate the existence of an homeostatic control of Omp synthesis/envelope composition.

4- Genetic approach applied to the study of the σ^E-dependent sRNA *rybB* gene rendered two groups of mutantions on promoter region and on the structural part. Within the latter, mutations mapped in three different regions: 5' end, center and 3' end of the RybB molecule.

5- A genetic analysis of the σ^E-dependent sRNA RybB allowed identification of the structural elements implicated in the function of this sRNA at the molecular level. The same analysis, when applied to the 5'UTR of two RybB target mRNAs, lead to a complete picture of sRNA-RNA inetractions. The pleiotropic quality of RybB regulation was found to be based upon recognition of an heptameric "seed"

sequence in 8 out of the 9 mRNA targets known to date. That seed was found to be duplicated in some of the target mRNAs. A new member of RybB regulon was described.

6- An sRNAs locus was shown to constitute an insertion hotspot for lateral DNA transfer thus contributing to genetic diversity. A further consequence of this DNA integration was shown to be the modification of the expression of the sRNA gene where the integration event occurred.

Agradecimientos

"Eh mentalizarze y quererze de verdá, zano, ze cuerpo, tené potencia, zé felí, quererte tu mihmmo a querete tu musho, porque quiere también ar que tiene a tu lao, y todo zale de verdad, de deporte."

Manuel Benítez "El Cordobés"

Los poquitos que vais a leer esto me conocéis y sabéis que tengo una memoria pésima; luego entenderéis cuánto me ha costado hacer una lista más o menos coherente de la gente gracias a la cual he conseguido terminar esta tesis. Como seguramente habrá olvidos, pido perdón por anticipado.

Echando la vista atrás, creo que debo extender mi lista de agradecimientos a mucho antes de comenzar la tesis, o siquiera la licenciatura; a los tiempos en los que mi profesor de primaria en el Joaquín Turina, José Luis Pérez, metía en el laboratorio a los tres raritos del colegio que no recibíamos clase de Religión, y dedicábamos esas "clases de Ética" a hacer experimentos llamativos y escuchar anécdotas científicas. Esas divertidas horas me despertaron un interés y una curiosidad fundamentales para encaminar estudios posteriores.

Algo más tarde, las clases de algunos profesores del Instituto Murillo influyeron decisivamente en mi perspectiva y mis preferencias. Por eso quiero agradecer al profesor de Lengua castellana y Literatura, Antonio Aranda, que me iniciase en el pensamiento científico riguroso y analítico. A los profesores Enrique Carreño, Lola Gago y Salvador López quiero dar las gracias por contagiarme su pasión por la Biología, la Fisiología y la Geología. Por último, quiero agradecer a Manuel Yoldi que ampliase sensiblemente mi visión y mis conocimientos sobre Química.

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