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Small RNA-mediated Regulation of Gene Expression in *Escherichia coli*

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Abstract

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Non-coding RNAs are highly abundant regulators of gene expression in all kingdoms of life that often play important roles in vital cellular functions. In bacteria, small regulatory RNAs (sRNAs) usually act post-transcriptionally by regulating mRNAs through base pairing within ribosome binding sites (RBS), thereby inhibiting translation initiation.

tisB encodes a toxin, TisB, whose synthesis is controlled by the sRNA IstR-1. Intriguingly, IstR-1 base pairs far upstream of the RBS but nevertheless inhibits translation initiation. The *tisB* mRNA is unusual in that ribosomes cannot access the RBS directly, but instead need an unstructured upstream region. This is precisely where IstR-1 exerts its inhibitory effect. We propose this region to serve as a ribosome loading site (standby site) which permits ribosomes to overcome the obstacle of inhibitory RBS-containing structures. Sequence-independent ribosome binding to the standby site allows for efficient relocation to the RBS structure when it is transiently open. Thus, standby sites are translation enhancer elements.

I also characterized TisB-mediated toxicity. The hydrophobic protein TisB is targeted to the inner membrane and causes damage. This decreases the intracellular ATP concentration and entails decreased replication, transcription and translation rates. It is likely that this toxin is involved in multidrug tolerance under certain conditions.

We identified the sRNA MicF as a negative regulator of *lrp* expression. Lrp is a global transcription factor that controls genes involved in amino acid metabolism and transport of small molecules. Interestingly, Lrp also downregulates MicF. Thus, this study established that the mutual downregulation of MicF/Lrp creates a positive feedback loop which gives a switch-like behavior important for fast adaptations.

Keywords: Small RNA, non-coding RNA, antisense, translational control, ribosome standby, toxin-antitoxin, IstR, TisB, tisAB, MicF, Lrp

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List of Publications

Publications I-III

This thesis is based on the following papers, which are referred to in the text as Paper I-III.

I *Darfeuille, F., *Unoson, C., Vogel, J., and Wagner, E.G.H. (2007) An antisense RNA inhibits translation by competing with standby ribosomes. *Molecular Cell*, 26, 381-392

II Unoson, C., and Wagner, E.G.H. (2008) A small SOS-induced toxin is targeted against the inner membrane in *Escherichia coli*. *Molecular Microbiology*, 70(1), 258-270

III *Holmqvist, E., *Unoson, C., Reimegård, J., and Wagner, E.G.H. (2010) The small RNA MicF targets its own regulator Lrp and promotes a positive feedback loop. Manuscript

*Shared first authorship

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Some of the results presented in this thesis are not included in the publications listed above

Additional publications

Unoson, C., and Wagner, E.G.H. (2007) Dealing with stable structures at ribosome binding sites. *RNA biology*, 4:3, 113-117 (point of view)

Contents

Abbreviations

sRNA	Small RNA
ncRNA	Non-coding RNA
UTR	Untranslated region
TIR	Translation initiation region
ORF	Open reading frame
SD	Shine-Dalgarno sequence
RBS	Ribosome-binding site
30S	Small ribosomal subunit
50S	Large ribosomal subunit
70S	Ribosome (30S + 50S)
TF	Transcription factor
TA	Toxin-antitoxin

Introduction

A historical view of gene regulation and RNA research

The central dogma in molecular biology states that DNA, the genetic material in most organisms, is transcribed into messenger RNA (mRNA), which acts as a blueprint by being subsequently translated into protein(-s). Historically, RNA was only considered to be important as an information-carrying intermediate between DNA and proteins, and as transfer RNA and ribosomal RNA involved in protein synthesis, but not to have any active/regulatory function. This view has changed dramatically in the last decades. Today, we know that RNAs can carry out many functions in the cells, such as catalysis and gene regulation.

In the early 1980s, the first catalytic RNAs were discovered; the ribonucleolytic component of RNAse P, M1 RNA, required for tRNA maturation, and the self-splicing group I intron of *Tetrahymena* (1,2). These findings resulted in the Nobel Prize in Chemistry 1989 awarded to Sidney Altman and Thomas Cech.

Jacob and Monod (3) were the first scientists who suggested that gene regulation in bacteria could be carried out by RNA. In 1981 the two first regulatory RNAs which employed the antisense mechanism (sequence complementarity to another RNA molecule) were discovered in bacteria. They were shown to be involved in copy number control of plasmids in *E. coli* (4,5). These were the first examples that showed that antisense RNAs could be bona fide regulators.

Today, many different non-coding RNAs (ncRNAs; not coding for proteins) are known in eukaryotes and prokaryotes. In addition to the previously known ribosomal RNAs, transfer RNAs and other so-called housekeeping RNAs, we now have a growing list of RNAs that act through an antisense mechanism to regulate gene expression. In eukaryotes, processes such as gene silencing and developmental regulation all largely depend on such regulatory RNAs (6). Bacterial regulatory RNAs were identified prior to their eukaryotic counterparts, with MicF in *Escherichia coli* being the first to be characterized (7). This opened up a whole new field; that of so-called small RNAs (sRNAs) in bacteria. Most sRNAs base pair to translation initiation regions (TIR) in the 5' UTR of mRNAs to affect translation and/or mRNA stability (8).

Two classes of eukaryotic regulatory RNAs that have expanded our view on RNA-mediated gene regulation/defense mechanisms are miRNAs and siRNAs (9). RNAi is a eukaryotic defense system, where invading or introduced dsRNA is processed into short, ≈22 base pair effector molecules, the short interfering RNAs (siRNAs). This is carried out by the enzyme Dicer, a member of the RNase III family (10). One strand of the siRNAs is incorporated into the RNA-induced silencing complex (11). This "active" strand guides the complex to the target RNA by sequence complementarity, leading to cleavage of the target RNA (9). RNAi is now frequently used as a powerful technique to knock down genes. The discovery of RNAi resulted in the Nobel Prize in Physiology or Medicine 2006 to Andrew Fire and Craig Mello (12).

The microRNA (miRNA) pathway is in some aspects similar to the RNAi pathway, but miRNAs are chromosomally encoded, processed in the nucleus, and regulate endogenously expressed mRNAs in the cytoplasm. miRNAs, at least in animals, usually exhibit nonperfect complementarity to target sequences within the 3' UTR of mRNAs (13,14). The base pairing of an miRNA to its target leads to RNA degradation or translational inhibition, depending on non-perfect or perfect target matches (15). Recent findings have also revealed an miRNA-mediated activation of mRNAs (16). If the base pairing between the miRNA and the mRNA contains mismatches – as usually in animals – an miRNA may target several mRNAs. miRNAs are considered to be important regulators of gene expression in animals and plants, affecting numerous pathways and functions (6,17).

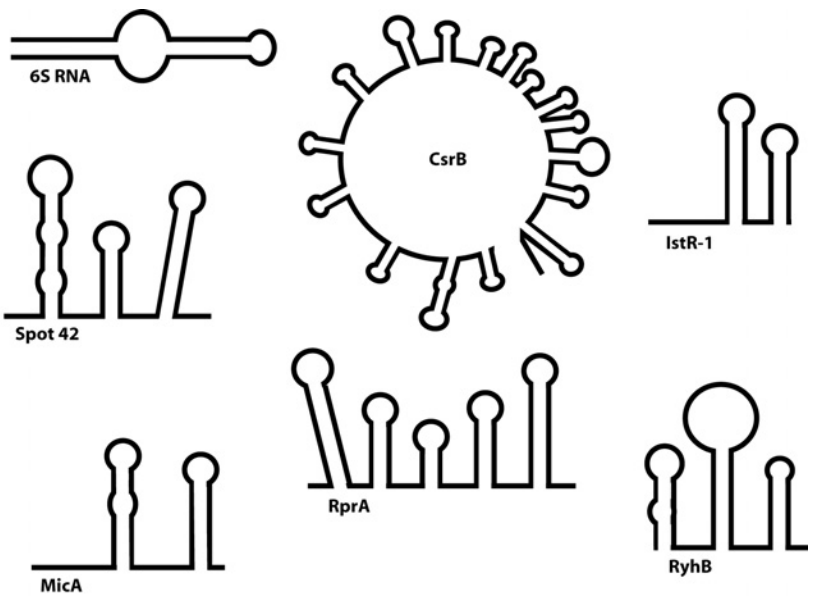


Figure 1. Examples of sRNA structures

Small RNAs in *Escherichia coli*

The diverse class of bacterial sRNAs has received much recent attention. Before the year 2001, with exception of the housekeeping sRNAs (tmRNA, RNase P M1 RNA, 4.5 S RNA) and the antisense RNAs encoded by accessory elements, only a few chromosomally encoded sRNAs were known in *Escherichia coli*. MicF was identified as an antisense RNA that regulates the expression of the outer membrane protein OmpF at the post-transcriptional level (7). 6S RNA was found already in 1967 (18) but its function remained elusive for many years. In 2001, the sRNA field expanded rapidly through genome-wide searches, resulting in the identification of many new sRNAs through either bioinformatical searches or microarray studies, and later also through shotgun cloning of size-fractionated RNAs (19-22). By 2003, approximately 60 sRNA had been identified, and – based on the assumption that most of these acted by antisense mechanisms – target searches were initiated to identify their functions. More than one third of the about 90 sRNAs known in *E. coli* today have now been characterized. sRNAs have been established as important regulators of gene expression in bacteria far beyond *E. coli* (23,24). It appears that the majority of the sRNAs are stress-related, helping bacteria to respond to changes in the environment (25-39). There is an ever-increasing number of identified sRNAs in many different bacterial species due to e.g. advanced multilayered computational searches, deep sequencing and tiling arrays (40-43).

sRNAs are in the size range of 50 to 300 nucleotides and display very distinct secondary structures consisting of one to several stem loops (Figure 1). They are commonly transcribed from their own promoters and have Rho-independent terminator stems. The activity of sRNAs in the cell is mainly determined by their synthesis rate, their structural features, their stability and processing (8).

Almost all sRNAs seem to act though an antisense mechanism by base pairing to the mRNA target(s). In many cases Hfq, a hexameric protein with RNA chaperone activity, is required for sRNA-mediated regulation. The majority of sRNAs inhibits or activates translation by either blocking, or rendering the TIR accessible for, translation initiation. Conversely, sRNA binding may also induce target RNA degradation, and combinations of translation effects and enhanced degradation are commonly found. There are also cases where the sRNA base pairs to its target in regions distant from the TIR and yet affects translation (see below). Only a few sRNAs have proteins as targets. This is reviewed in (44).

The sRNA can either have single or multiple targets. Conversely, a single target can also be regulated by either one or a few sRNAs. Different sRNAs often respond to different environmental stimuli. These are features of major benefits for sRNAs that participate in networks/regulons to coordinate the response to multiple signals.

About one third of all sRNAs characterized in *E. coli* targets outer membrane proteins (OMPs), generating an sRNA – OMP network important in remodeling of the outer membrane under appropriate conditions (45,46) (Figure 2). Such a network provides a dense overlapping regulatory web where multiple signals are integrated (47). sRNAs have been shown to be very efficient at filtering noise due to strong input signals, such as e.g. membrane stress (48,49). This sRNA – OMP network links both nutrient availability and sugar metabolism to membrane stress (50-53) to preserve membrane integrity under various conditions.

In general, the small bacterial RNA regulators control gene expression involved in many pathways, from carbon utilization and sugar metabolism, membrane composition and stress responses, to toxicity and virulence (54). Bacterial sRNAs are in many aspects functionally similar to eukaryotic miRNAs. See Table 1 for the so far characterized sRNAs in *E. coli*.

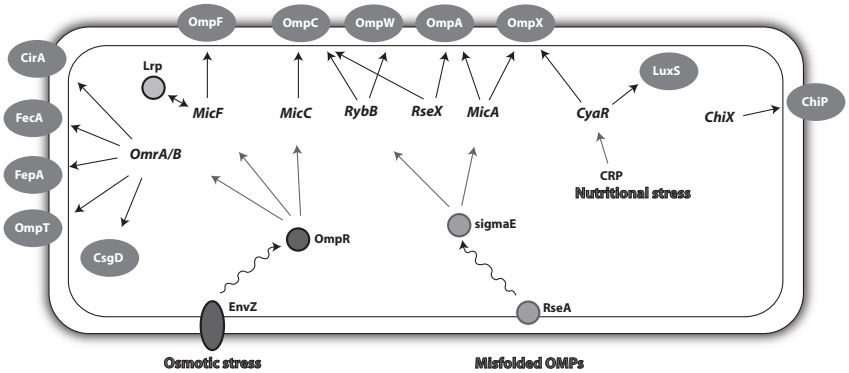


Figure 2. The sRNA – OMP network. The outer membrane proteins are represented by the filled circles located in the outer membrane. Different sRNAs targets different OMPs, as depicted by the arrows. The stress responses, sigma factors, transcription factors and other signals identified as regulators of sRNA expression levels are also shown.

Antisense mechanisms

Most of the plasmid-encoded sRNAs are cis-encoded, i.e. they are divergently transcribed within the target gene, which generates a perfect complementarity. In contrast, most sRNAs derived from chromosomes are trans-encoded. They do not overlap the target gene, resulting in a partial complementarity to their targets (Figure 3), enabling them to target more than one mRNA. Usually only a core of nucleotides is critical for the regulation (in Paper III a single point mutation in the interaction region abolished regulation).

Both the intracellular concentration of the RNAs and their binding rate constants are important parameters for the efficiency of the antisense effect. The binding rate constants (association rate constant, k_{app}) are usually in the order of ~ 10^5 -10^6 M^{-1} s^{-1} for cis-encoded sRNA interactions (55), but much lower for their trans-encoded counterparts (e.g. 10^4 M^{-1} s^{-1} for MicA-*ompA* mRNA, (56)). The initial regions of interactions between the sRNA and its target mRNA need to be single-stranded/unstructured for base pairing to occur. It is most often either one of the loops in the sRNA (e.g. OxyS-*fhlA* mRNA) or the 5' tail of the sRNA (e.g. MicA-*ompA* mRNA (56) or IstR1-*tisAB* mRNA (25)) that is responsible for the initial interaction and which likely is rate-determining.

The efficiency of sRNA regulation is dependent on binding rate rather than binding affinity (57,58). If the on-rate constant is quite low, as is often the case for the trans-encoded sRNAs, Hfq may be required to enhance the binding (59).

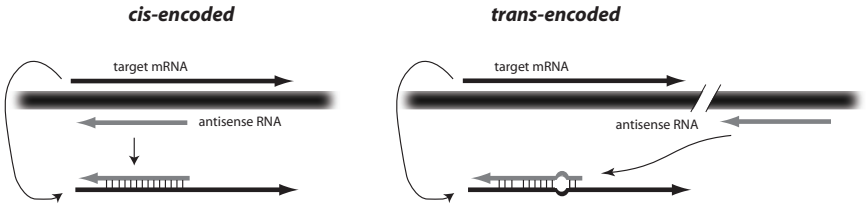


Figure 3. Cis- versus trans-encoded sRNAs. Cis-encoded sRNAs are encoded on the opposite strand of their targets, whereas trans-encoded sRNAs are encoded elsewhere on the chromosome.

Translation inhibition by targeting the TIR

The 30S ribosomal subunit requires the TIR (translation initiation region), including the Shine-Dalgarno (SD) and the start codon, on the message to be structurally accessible in order to bind and initiate translation. Once the 30S has formed the ternary complex, involving base pairing between SD and anti-SD, and fMet-$tRNA^{fMet}$ base paired to the start codon, 50S subunit joining forms the translation-competent 70S ribosome, and translation can start. These steps require a set of initiation factors (IF1-3) needed for initiator met-tRNA selection, start site selection and subunit association at the start codon. The major determinant for the efficiency of translation initiation is the SD-region – its sequence, its spacing to the start codon, and most importantly the presence or absence of inhibitory structures. In the translation process, consisting of translation initiation, elongation, and termination, translation initiation is the rate-limiting step (60,61).

The majority of sRNAs binds to the TIR on mRNAs, occluding the ribosome binding site (SD and/or AUG), thereby preventing ribosomes from

initiating translation. This is the most frequently found mechanism for sRNA regulation (Figure 4A and Table 1). Small RNA base pairing may inhibit translation initiation by binding inside the ORF, down to the fifth codon (62). This means that there is a competition between the sRNAs and the initiating ribosomes in this specific region in the mRNA. The sRNA must bind to the TIR before the ribosome has formed a stable initiation complex, to block translation. Since bacteria usually exhibit coupled transcription-translation, the RNA polymerase (RNAP) transcribes the message, and the ribosome enters to initiate translation while transcription is still on-going.

Degradation versus translation inhibition

As a consequence of prohibited ribosome recruitment due to sRNA binding, the naked mRNA becomes vulnerable to RNase E degradation (RNase E is the major endoribonuclease in *E. coli)*. The effects of sRNAs on target mRNA levels are indeed well-supported in the literature (63). A question that has arisen from this concerns whether it is translation inhibition or degradation of the message that is the predominant effect of the sRNA-mediated regulation. By using an RNase E-deficient strain, Aiba and co-workers could decouple degradation from translation inhibition. They showed that the sRNA SgrS could inhibit translation of *ptsG* mRNA without any degradation occurring. This indicated that, at least for this case, translational inhibition is the primary effect, and rapid mRNA degradation is a secondary consequence (64). *In vivo* sRNA-dependent inhibition of translation causes degradation of the RNA duplex. Since both the message and the sRNA appear to be degraded (65), there is no recycling of sRNA, i.e. they act stochiometrically.

As has been demonstrated recently, degradation can also be the main or even only pathway of inhibition. MicC, an sRNA involved in membrane stress, targets *ompD* mRNA in *Salmonella typhimurium* by base pairing far inside the coding region. This binding does therefore not interfere with translation initiation but instead promotes fast decay of the sRNA-mRNA duplex in an RNase E-dependent manner (66).

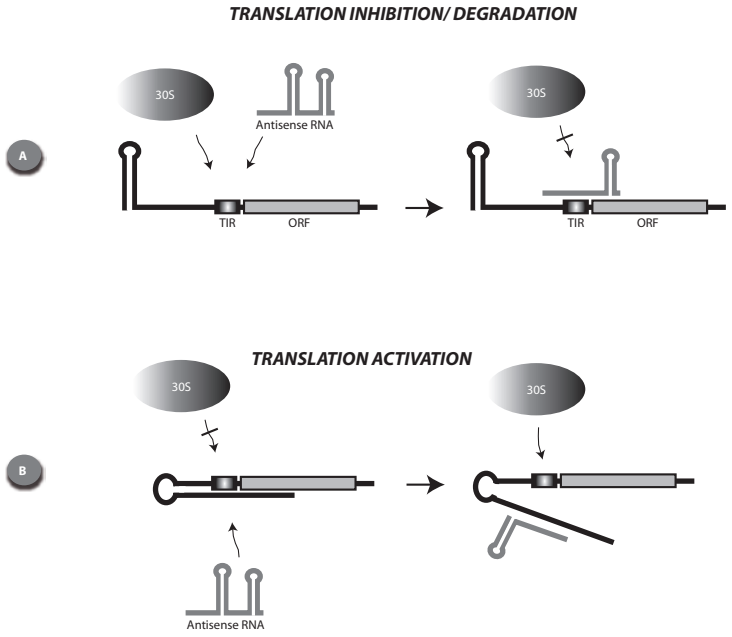


Figure 4. sRNA base pairing affects the target translation and/or degradation. sRNA binding may induce both A) translational repression and B) translational activation depending on the location of sRNA binding site and the internal structure of the mRNA target.

Translation activation

Even though translational repression is the most common regulatory effect of sRNAs, there is also a class of upregulatory sRNAs that activate translation of targets. The majority of these mRNA targets have their TIR sequestered in an internal structure, and an sRNA is required to open up this structure by an anti-antisense mechanism (Figure 4B). The most extensively studied sRNA-mediated upregulated target in *E. coli* is *rpoS* mRNA, which is activated by three sRNAs; DsrA, RprA and ArcZ (67-70). The *rpoS* gene encodes the major stress sigma factor, σ^S. DsrA, RprA, and ArcZ liberate the internal inhibitory structure by base pairing to the strand that otherwise sequesters the TIR, thereby disrupting this inhibitory structure. The three different sRNAs are expressed under different conditions, thereby promoting *rpoS* expression when needed.

Another case to which activation applies is RNAIII. In addition to acting as a negative regulator and an mRNA, RNAIII also activates *hla* mRNA, coding for a major virulence factor. Also here the mechanism is liberation of an internal structure sequestering the TIR, thereby enhancing translation of the message (71).

sRNAs targeting non-TIRs to control translation

Some sRNAs base pair to their mRNA targets in regions outside the TIR and prevent translation (Figure 5). How do those non-TIR targeting sRNAs operate? There are now a few examples of such sRNAs which all control translation by distinct regulatory mechanisms.

IstR-1 binds ~100 nts upstream of the *tisB* TIR (TisB is a toxin involved in the SOS response) to inhibit translation initiation, by blocking ribosome recruitment. The ribosomes compete with IstR-1 for a ribosome loading site/standby site present elsewhere on the message (72). I will describe this mechanism in more detail below (Present Investigation, Paper I).

GcvB is an sRNA that targets several mRNAs encoding proteins involved in the ABC uptake systems for amino acids and peptides. GcvB binds to A/C-rich sequences, both off and on TIR regions, within several target mRNAs. These A/C-rich regions serve as translational enhancer elements, and GcvB therefore inhibits translation by blocking access to these elements (73).

Another pair of sRNAs that act off TIR are OmrA and OmrB, which regulate *csgD* mRNA. CsgD is a transcriptional activator of curli genes. OmrA/B bind far upstream of the *csgD* SD to inhibit translation. It is clear that this region is essential for regulation of the message, but the detailed mechanism of regulation is not yet fully understood (74).

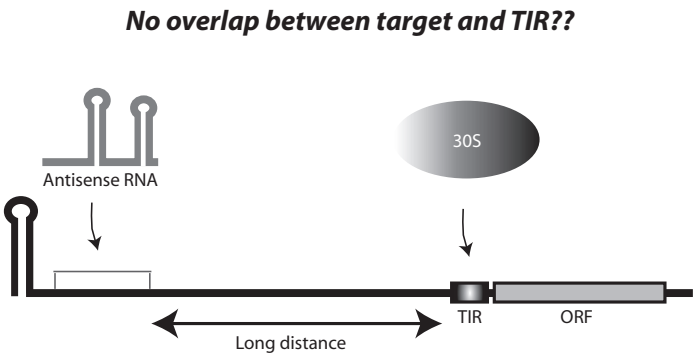


Figure 5. sRNAs may bind to regions distant from the TIRs. sRNAs may control translation efficiency without binding to the TIR, thus without interfering with initiating ribosomes

Protein targeting sRNAs

Only three protein-targeting sRNAs have so far been identified in *E. coli*: 6S RNA and CsrB/C. They are conserved in many bacterial species and they employ two very unique regulatory mechanisms.

The highly abundant 6S RNA accumulates in stationary phase to shift the RNA polymerase (RNAP) σ-factor usage from σ^{70} to σ^{S} to start transcription of the stationary phase genes (75). 6S RNA has a secondary structure that resembles an open promoter (Figure 1). This open-promoter-like RNA traps the σ^{70}-RNAP in a stable complex in stationary phase, inhibiting the transcription of many σ^{70}-dependent genes and forcing the shift to transcription of σ^{S}-dependent genes. The stable 6S RNA-σ^{70}-RNAP complex is reversed when the levels of nutrients or nucleotides increase. 6S RNA then functions as a true (DNA) promoter coding for a short RNA product (pRNA) and, during outgrowth, the trapped σ^{70}-RNAP transcribes this pRNA. This event somehow promotes σ^{70}-RNAP to be released from 6S RNA, which then permits the transcription of σ^{70}-dependent genes again (76). This is a so far very unique sRNA-mediated regulation where the sRNA mimics a DNA template for transcription.

CsrA is an RNA binding protein that post-transcriptionally regulates translation and/or stability of mRNAs involved in carbon metabolism. CsrA activity is regulated by two functionally redundant sRNAs: CsrB and CsrC. CsrB out-titrates CsrA, by mimicking the region that CsrA normally binds to on its targets. Up to 18 CsrA proteins may be sequestered by one CsrB sRNA. The Csr system is involved in e.g. carbon metabolism, motility and biofilm formation in *E. coli*, in quorum sensing in *Vibrio cholerae*, in epithelial cell invasion in *Salmonella enterica*, and its homologous Rsm system is involved in e.g. quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and in fungal plant disease suppression in *Pseudomonas fluorescens* (77).

Odd sRNA characteristics

Small RNAs are classified as non-coding RNAs (not containing any open reading frame (ORF)), but this is not true for all sRNA, as there are of course exceptions.

The extensively studied and characterized RNAIII in *Staphylococcus aureus* is such an exception. RNAIII is a key regulator of virulence. This long (514 nt) sRNA acts both as a negative and positive regulator by antisense mechanisms, and in addition acts as an mRNA. It contains an ORF encoding δ–hemolysin. The 5' end of the transcript contains the ORF and several non-coding domains are responsible for the regulatory functions (71,78-81).

In *E. coli* the "exception" is SgrS. This sRNA is expressed during glucose-phosphate stress and negatively regulates the *ptsG* mRNA, encoding a major glucose transporter (27). It was found that SgrS also contains a short ORF, coding for a peptide SgrT. Unexpectedly, SgrT and SgrS have additive

roles during glucose-phosphate stress to quickly shut down glucose transport (82). Thus, Sgrs and RNAIII are dual-function RNAs.

Even though most of the chromosomally encoded sRNAs are trans-encoded, some sRNAs are cis-encoded and exhibiting perfect complementarity to their targets. This applies to e.g. GadY (33), SymR (83), and many sRNAs that act as antitoxins (84,85).

Recently, a number of long (~1 kb) cis-encoded transcripts have been identified in several bacteria (40,41,86). The importance of these long, putative antisense RNAs still remains obscure, but a few of them appear to have regulatory effects on their respective cis-encoded targets (87).

In the following sections, the main sRNA mechanisms will be described, as well as their regulatory effects.

Hfq is an important player in sRNA-mediated regulation

Hfq is a highly abundant homohexameric Sm-like protein, conserved in many bacteria, which is important for many trans-encoded sRNAs (88). Many sRNAs lose their regulatory ability in Hfq deletion strains. Hfq has three main roles; it can affect the metabolic stability of both sRNAs and mRNAs (89-91), it may promote sRNA-mRNA annealing (92-94), and its chaperone activity may unfold RNA structures to facilitate RNA-RNA interactions (95). Hfq is essential for many cases of sRNA-mediated regulation. Its absence has therefore global consequences, such as stress-sensitivity and loss of virulence (96-98). It was recently reported that RNAs actively exchange on Hfq (Fender, 2010, unpublished). Rapid cycling, driven by high intracellular concentrations of binder RNAs, would solve the paradox that RNAs bind very tightly to Hfq, but RNAs can still access Hfq very quickly (sRNAs generate fast regulatory effects, but the Hfq pool is limited). This is important since most sRNAs are induced due to stress, i.e. under conditions when the cell needs rapid adaptation. sRNA-mRNA interactions that are perfect and contiguous are usually Hfq-independent.

Table 1. Effects of *E. coli* sRNA on their targets

mRNAs						proteins	response
pos/neg	sRNA	target/-s	T/C	TIR	Hfq	target	
neg	CsrB/CsrC					CsrA	carbon metabolism
neg	6S RNA					Es70	stationary phase survival
pos	GlmY					Yhbj?	sugar metabolism
neg	MicA/MicC/MicF RybB/OmrA/OmrB CyaR/ChiX/RseX	omp's	T	+	+		membrane stress
neg	Spot42/SgrS		T	+	+		sugar metabolism/stress
neg	OxyS	rpoS, fhlA	T	?/+	+		oxidative stress
neg	GcvB	ABC transporters	T	-/+	+		amino acid transportation
neg	IstR1/SymR	tisAB, symE	T/C	-	-		SOS response
neg	DicF	ftsZ	T	+	?		cell division
neg	MgrR	eptB, ygdO	T	+	+		LPS modification[1]
neg	RdlD	ldrD	C	-	-		
neg	DsrA	hns	T	+	+		thermal stress
neg	FnrS	several..	T	+	+		anaerobic metabolism
neg	ArcZ	several..	T	+	+		general stress
neg pos	RyhB	several.. shiA	T	+ *	+ +		iron homeostasis
pos	GlmZ	glmS	T	*	+		sugar metabolism
pos	DsrA/RprA/ArcZ	rpoS	T	*	+		general stress
pos	GadY	gadX	C		+		acid stress

Pos: positive regulation, neg: negative regulation, T: trans-encoded, C: cis-encoded, Hfq: + Hfq-dependent, - Hfq-independent, TIR: + binding to TIR, * rendering the TIR open, LPS: lipopolysaccharide. This list is not complete and only covers sRNAs in *E. coli*. References are: (25,27,28,33,37-39,45,46,50,52,70,73,75,77,99-104)

CRISPR RNAs inhibit uptake of foreign DNA

Another new class of regulatory RNAs was recently discovered; CRISPR RNAs (crRNAs) (105). They are the specificity determinants of a new bacterial and archaeal RNA-based defense system, which mechanistically is not yet fully understood. There is great variability in this system in different bacterial species and in archaea. CRISPR provides resistance to bacteriophages and prevents plasmid conjugation. CRISPR loci encode a DNA array consisting of a leader followed by multiple identical repeat and unique spacer units. Some spacer sequences correspond to phage or plasmid sequences. Several CRISPR-associated (CAS) genes are found adjacent to the CRISPR arrays. The proposed model is the following; The CRISPR DNA array is transcribed into a long RNA, which is processed to shorter RNAs (crRNAs), corresponding to a repeat-spacer unit, by (in *E. coli*) the Cascade complex consisting of five CAS proteins (106). These crRNAs are later directed to target phage or plasmid DNA (or RNA) by sequence complementarity to prevent invasion of foreign DNA (106-108). New phage sequences can be inserted into the present CRISPR array during infection

and these then confer resistance to that specific phage and other phages containing the same sequences (107).

The CRISPR defense system is akin to acquired immunity. It builds up over time and enables bacteria and archaea to survive e.g. phage invasions, somewhat analogous to eukaryotic RNAi (105,109).

TA modules employ sRNAs as antitoxins

Small RNAs may act as antitoxins in toxin-antitoxin (TA) systems, where they repress expression of proteins which are toxic at high levels. Many TA loci are present in free-living prokaryotes and in many of their plasmids (110). In *E. coli*, more than 20, and in *Mycobacterium tuberculosis* more than 80 TA loci have been identified (85,110-113). Most TA systems are present in multiple copies. The toxins are typically stable proteins that inhibit an important cellular function when induced by a specific stress, whereas the more unstable antitoxin may be an sRNA or a protein. The antitoxins counteract toxicity at either the post-transcriptional level, where an sRNA targets the toxin mRNA resulting in decay of the RNA duplex (type I TA), or at the post-translational level, where a proteic antitoxin binds to the toxin generating a non-functional toxin-antitoxin complex (type II TA). Many TA modules present on plasmids are involved in plasmid maintenance (84,114,115).

The difference in stability between the toxin and the antitoxin is crucial for the functioning of post-segregational killing systems in plasmids. When plasmids are present in cells, both the toxin and the antitoxin are simultaneously expressed, and thus toxicity is prevented. In plasmid free cells, both toxin and antitoxin are still present from the mother cell, but since the antitoxin is labile, it is degraded and the more stable toxin mRNA will be translated, resulting in toxicity. This results in cell death of the plasmid free cell, thus ensuring plasmid maintenance (84).

Less is known about the physiological roles of chromosomally-encoded TAs, even though they are quite abundant. MazF and RelE are two of the most well-studied type II toxins in *E. coli*. They cleave mRNAs to inhibit translation, with different specificities due to stress, e.g. nutrient starvation (116-121). Whereas most of the plasmid-encoded toxins lead to cell death once expressed, their chromosomal counterparts seem to be bacteriostatic. There is however a controversy regarding whether MazF causes cell death or cell stasis (117,122). In the following section I will focus on the type I TA pairs, i.e. the ones that use sRNA to regulate toxicity.

Hok-Sok

Of the many type I TA modules, Hok-Sok is the most extensively characterized one. This system is involved in plasmid maintenance (84). The *hok* gene encodes a small hydrophobic protein capable of *ho*st *k*illing (thereof the name). The *hok* mRNA is stable (half life of ~ 20 minutes) and Sok is an unstable cis-encoded antitoxin RNA (half life of ~ 30 seconds). The full-length *hok* mRNA is translationally inactive (123). This means that transcription is uncoupled from the translation for this message. A slow 3' trimming of *hok* mRNA occurs, which induces structural rearrangements of the message. A closer look at the *hok* message revealed a preceding/overlapping ORF, *mok,* whose translation has been shown to be required for Hok translation (translational coupling). Both the *hok* and the *mok* TIRs are sequestered in the full-length message, but the *mok* RBS becomes accessible in the 3' trimmed and refolded message. The antisense RNA, Sok, binds to the TIR of *mok* to inhibit translation initiation, which prevents translation of Hok and entails RNAse III-dependent decay of the transcript (124). Since Sok is unstable, plasmid loss rapidly depletes the available Sok pool, thus eventually resulting in Hok translation. Hok expression kills cells in a manner analogous to that caused by the holin proteins produced by phages before cell lysis, and this specifically prevents the survival of plasmid free cells (125).

Chromosomally encoded type I TA systems

In *E. coli*, a few TA systems with RNA antitoxins have been characterized. Of these, two are involved in the SOS response, IstR1-TisB (25) and SymE-SymR (83), and three are most probably constitutively expressed, LdrD-RdlD (102), IbsC-SibC and ShoB-OhsC (126). Genomic searches have revealed many more type I TA modules, but they still await characterization (85). TA pairs seem to more frequently employ cis-encoded sRNAs as antitoxins, compared to other systems that employ sRNAs. Two trans-encoded antitoxin sRNAs are IstR-1 and OhsC RNAs, which are non-overlapping, divergently encoded (25,126). Many TA systems of both type I and type II are present in multiple copies. SymE-SymR will be briefly addressed below, and the TisB-IstR1 system will be more extensively described (Present investigations).

The cis-encoded antitoxin SymR regulates the translation of the SOS-induced toxin SymE (83). SymR base pairs to the *symE* SD to inhibit translation under non-DNA damaging conditions. SymE is transcriptionally repressed by LexA in the absence of SOS signals. It was suggested that SymE, when expressed, recycles RNAs after e.g. UV exposure by degrading damaged RNAs that otherwise could be lethal to the cell.

It is striking that almost all type I toxins are small hydrophobic proteins that target the membrane, with SymE being an exception (see Table 2). Overexpression of TisB, ShoB, IbsC, and Hok leads to membrane depolarization (125-127). This also applies to other type I toxins, such as the plasmid-encoded Fst and TxpA in *Bacillus subtilis* (128-130).

Table 2. Some characteristics of identified type I toxins in *E. coli*

Antitoxin (sRNA)	Toxin (Protein)	Size (aa)	Hydrophobic
IstR-1	TisB	29	Yes
SymR	SymE	113	No
OhsC	ShoB	26	Yes
SibC	IbsC	18-19	Yes
RdlD	Ldr-D	35	Yes
Sok	Hok	50	Yes

References are: (83,102,125-127)

Implications of structured TIRs

An important issue regarding sRNA-mediated control concerns the implications of structured TIRs. Ribosomes have difficulties initiating translating on mRNAs whose TIRs are structurally sequestered. The TIR needs to be at least partially open for the 30S ribosome to bind (to the SD sequence and the AUG). I.e., translation efficiencies generally increase as a function of decreased thermodynamic stability of the TIR. It is not uncommon that an inhibitory structure sequesters the TIR region. Nevertheless, translation may still occur at high rates (131). Bacteria have developed mechanisms to get around inhibitory structures, e.g. by employing cis-acting RNA elements, upstream ORFs and so-called ribosome standby sites.

One type of post-transcriptional control employs cis-acting regulatory RNA elements within the mRNA leader; so-called riboswitches (132,133). A riboswitch may adopt one of two mutually exclusive alternative structures, resulting in either up- or downregulation of the gene it precedes. These alternative structures can be induced by e.g. binding of metabolites. In gram negative bacteria, most riboswitches affect translation initiation, whereas they mostly affect transcription termination in gram positive bacteria (134).

Another class of cis-acting regulatory RNAs is the thermosensors (Figure 6A). Here translation is impeded by a stable structure encompassing the TIR in mRNAs. This inhibitory RNA structure is melted upon an increase in temperature, resulting in an open TIR, which permits translation. Temperature control is employed for e.g. expression of *rpoH*, encoding the major sigma factor under heat shock conditions (135), and for expression of *prfA* in *Listeria monocytogenes*, encoding a transcription factor involved in virulence at 37°C (136). Cis-acting regulatory RNAs may also be cold- or pH-controlled, which results in e.g. altered degradation of the mRNA at high temperatures, or altered pausing of RNA polymerase generating a translation-competent mRNA under high pH conditions (137,138).

In some cases, inhibitory structures are stable in most conditions, and ribosomes need help to access these TIRs. An ORF located upstream on the same message may solve this problem by translational coupling. The ribosome translates the preceding ORF, transiently opens the downstream TIR, and continues by reinitiation. This is possible since actively translating ribosomes can read through otherwise inhibitory RNA structures (Figure 6B).

The plasmid R1-encoded *repA* (encoding the replication initiation protein) and *hok* (encoding a toxin involved in plasmid maintenance) employ translational coupling (123,139). Both of them have an upstream ORF coding for a leader peptide necessary for translation. Since the *repA* RBS and the *hok* RBS are sequestered, translation efficiency is determined by the upstream RBSs. These in turn are subject to translational control by sRNAs: CopA and Sok, respectively (124,139). Translational coupling hass also been reported in ribosomal protein operons (140,141).

If a stable TIR RNA structure cannot be destabilized, translation initiation should be inhibited. The time window during which the RBS-containing structure becomes open as a consequence of the unfolding/folding equilibrium is too short to efficiently recruit a 30S subunit from the cytoplasm. Thus, the local concentration of ribosomes must be increased to ensure that ribosomes are in place near the TIR to rapidly access it upon transient opening. By introducing a sequence-nonspecific loading site (142) on the message, a ribosome may sit on standby/already close to the TIR for some time and shift into place once the TIR opens. This set-up implies that the diffusion rate of the ribosomes is no longer limiting and the ribosomal relocation rate may be close to the unfolding rate of the TIR structure. This ribosome standby concept, originally proposed by de Smit and van Duin (142), will be extensively described in paper I, since it applies to the post-transcriptional control of *tisAB*.

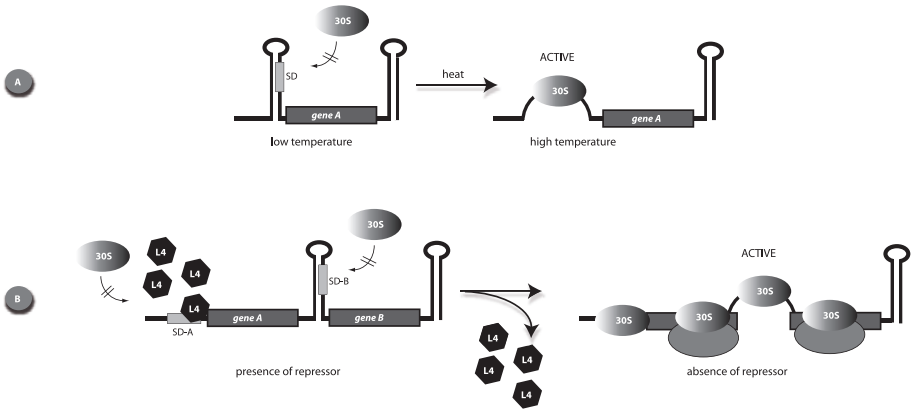


Figure 6. Two mechanisms to overcome inhibitory structures that repress translation initiation. A) Thermosensor regulation: the RBS is relieved from the internal structure at higher temperatures. B) Translational coupling: the α-operon is autoregulated by the ribosomal protein L4. The presence of L4 at the first RBS (SD-A) and an internal structure encompassing the second RBS (SD-B) on the polycitronic mRNA inhibits translation initiation at both TIRs. In the absence of L4, 30S may initiate translation at the derepressed SD-A. The actively translating ribosomes can then break the structure at the second RBS, and thus continue the translation of gene B.

Biological aspects: when and why to use sRNAs

Gene regulation occurs on several levels; on the DNA level (transcriptional), RNA level (post-transcriptional) and protein level (post-translational). Different combinations of regulators as a layered regulation, acting at the DNA, RNA or protein levels, gives different regulatory outcomes. Dynamic simulations have demonstrated that sRNA-mediated regulation generates a much faster response than transcription factor-based (TF) regulation (143). Since sRNAs act stochiometrically, the concentrations of sRNAs and target mRNAs, and their molar ratios, are critical for regulation. If an sRNA is in high excess over the target mRNA, it will efficiently turn on/off gene expression, whereas the sRNA has little effect when it is far from saturation (Figure 7, (48,144)). This behavior is due to a threshold linear response for sRNAs, in contrast to a linear response for TFs (48,144). The sRNA threshold property results in a less efficient regulation due to a small or transient input signal. sRNAs are better suited for transducing stronger and more persistent signals, such as membrane stress, resulting in a stronger and more reliable regulation compare to TFs (48,49). TFs and sRNAs may be suited for different biological tasks, responding to different signals. In stress responses, when the cells need a fast adaptation, sRNA usage may be advantageous, because of e.g. the “speed factor” (143,145).

Some genes need to be kept silent under certain conditions. Other genes, critical in multiple central cellular processes, also require an efficient regulation. A layered regulation is especially important for such genes, to ensure a tight and controlled regulation due to many different environmental stimuli.

One gene exhibiting such a layered regulation is *rpoS*, encoding the alternative sigma factor σ^S. *rpoS* needs to be expressed under many different conditions, such as stationary phase, osmotic imbalance, cold shock, and low pH (146). Hence, transcriptional, post-transcriptional and post-translational regulations all act in concert to ensure appropriate expression of the sigma factor (37,67-69,146).

Other cases with a similar rationale apply to toxins. Toxicity must be prevented unless a program requires it. Several sRNAs, encoded both by plasmids and the chromosome, act as antitoxins by inhibiting the translation of toxin mRNAs (84).

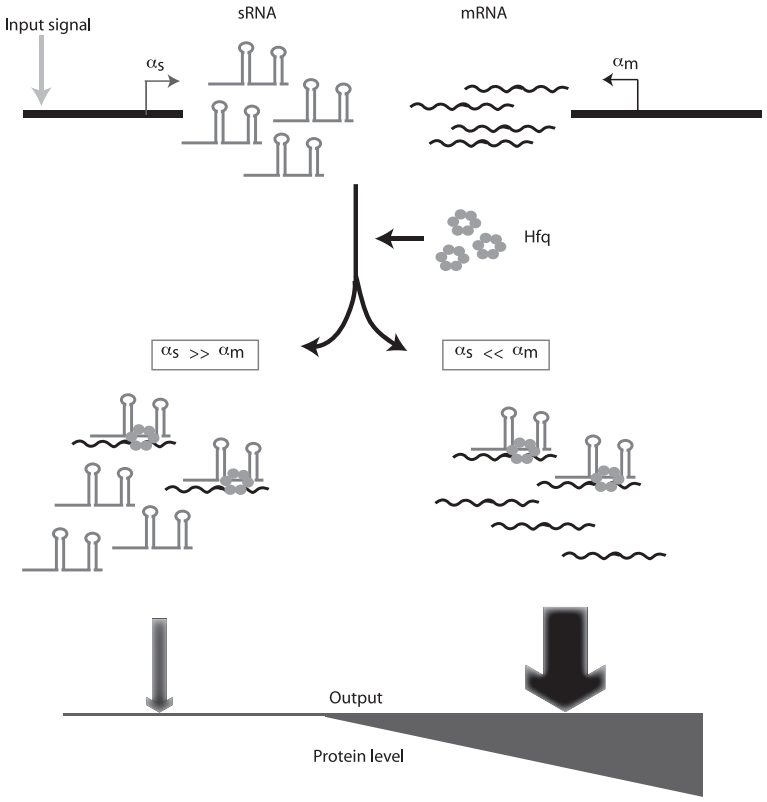


Figure 7. sRNAs act stochiometrically on their targets. Depending on the concentrations of RNA molecules present, different regulatory effects are obtained, where α_s and α_m is the transcription rate of the sRNA and mRNA, respectively. The regulatory effect is manifested as the generated protein levels. This illustration is based on a figure in Mehta et al, 2008 (48).

The present investigation

istR-tisAB; a new toxin-antitoxin module (Papers I-II)

The SOS response is primarily a response to DNA damage which induces post-replication DNA repair systems. SOS-involved genes are negatively controlled by the master regulator LexA. Once many gaps in the DNA occur, the dimeric recombination protein RecA becomes auto-cleaved, which induces autocleavage and inactivation of the dimeric repressor protein LexA. This results in a derepression of SOS genes, which encode proteins of various biological functions, e.g. repair proteins and protein chaperones (147).

The SOS-induced toxin TisB, identified in 2004 (25), is controlled by the 74 nt-long sRNA IstR-1. IstR-1 was the first SOS-associated sRNA to be described and characterized. TisB is encoded by the LexA-repressed *tisAB* mRNA, whereas the divergently transcribed IstR-1 is constitutively expressed. IstR-1 base pairs 100 nts upstream of the *tisB* SD to inhibit translational initiation by an, at the time, unknown mechanism. It was intriguing that IstR-1 could base pair to a region far upstream of the *tisB* SD to affect translation. The presence of an ORF, *tisA*, in this target region, suggested a mechanism for inhibition which however was subsequently ruled out genetically and biochemically; TisA is neither translated nor involved in toxicity (25, 72). Interaction between IstR-1 and *tisAB* mRNA is based on a contiguous complementarity of 21 bp which entails RNase III cleavage (RNase III cleaves dsRNAs of approximately two helical turns/~ 20 nucleotides (148)) and thereby translational inactivation of the mRNA. This is one of few known sRNA-mRNA interactions (i.e. for trans-encoded sRNAs) that are Hfq-independent. IstR-1 prevents TisB toxicity in the absence of the SOS response. Under SOS conditions, induction of *tisAB* overrides IstR-1, and TisB is translated, resulting in growth defects. The constitutive high-level expression of IstR-1 may also lead to a fast off-switch when TisB expression is no longer required. The TisB toxin was uncharacterized prior to these studies.

In the study from 2004 (25), three different *tisAB* mRNA species were detected on Northern blots; the primary *tisAB* mRNA, the RNase III-cleaved mRNA and one additional *tisAB* mRNA species. This was noted, but the full implications of this remained unclear until later.

The *istR-tisAB* locus also encodes a second sRNA, IstR-2 (140 nt, LexA-controlled) which is not involved in *tisAB* mRNA regulation, even though IstR-1 and IstR-2 share the region of complementarity to *tisAB* mRNA (different promoters, same 3' end). We believe that IstR-2 has other, not yet identified targets, most likely within the SOS regulon (data not shown). Figure 8 shows the locus with all identified *tisAB* mRNA species.

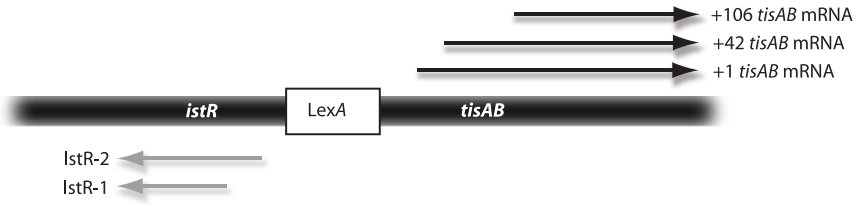


Figure 8. The *tisAB* locus, with all the transcribed RNA species identified

Post-transcriptional regulation of *tisAB* (Paper I)

tisAB is tightly regulated, both transcriptionally (LexA controlled) and post-transcriptionally. An unexpected complexity of regulation at the post-transcriptional level was uncovered in the study presented below. This includes both inhibitory intramolecular RNA structures, the role for an unstructured RNA segment, and IstR-1 control.

In order to mechanistically study the regulation of *tisAB* expression, we employed *in vitro* experiments. The main issues we wanted to investigate; what was the purpose of the three different variants of *tisAB* mRNA present *in vivo* (detected on Northern blots, (25)) and how can IstR-1 regulate *tisAB* mRNA by base pairing far upstream of the *tisB* TIR.

Only one out of three mRNAs is active

The three detected mRNA species were the following: first, the primary transcript of the mRNA (+1), second, an endonucleolytically cleaved/processed mRNA (+42) and third, the IstR-1-dependent RNase III-cleaved mRNA (+106) (Figure 8). Surprisingly, results from an *in vitro* translation assay showed that only the +42 mRNA was translated, indicating that the primary message has to be processed in order to become active for translation. This was confirmed by toe-printing assays, in which initiation complex formation was monitored (149); a toe-print at the *tisB* SD was only obtained on +42 mRNA but neither on +1, +106, nor +42 when bound by IstR-1. Note that all of these three mRNA species contained the entire *tisB* ORF. From this we could conclude that only one *tisAB* mRNA species, +42, was active, i.e. translational-competent. What remained obscure was why

this transcript was active and not the others – they all contained the *tisB* ORF, what was the difference? Since IstR-1 base paired to a region approximately 100 nt upstream of the *tisB* SD to inactivate the mRNA, this region appeared to be required for activity.

Translational coupling or structural rearrangements?

Two plausible scenarios could be envisioned. The first one would suggest that IstR-1 base pairs far upstream of the *tisB* SD to block a potential translational coupling event, and the second one would involve IstR-1-induced secondary structure changes in the mRNA that would prevent TisB translation. The *tisAB* mRNA contains two ORFs; *tisA* and *tisB*, and IstR-1 binds to the putative TIR of *tisA*. Earlier *in vivo* studies had shown that *tisB* was the ORF encoding the toxin (25). Mutational studies and differential labeling showed that *tisA* was neither expressed nor required for *tisB* expression, thus ruling out translational coupling. The second model was tested by extensive structural mapping (150) of the three different *tisAB* mRNA species, with and without IstR-1 bound. Strikingly, the 3' segments of all mRNAs were indistinguishable in secondary structure (this part includes the *tisB* TIR and ORF) whereas the 5' segments differed. The 5' parts of the two inactive mRNA species were either structurally sequestered (+1), or cleaved off and thus shorter (+106). By contrast, processing of the primary *tisAB* mRNA led to refolding to generate a long single-stranded region in the 5' end. The *tisB* TIR in all three RNAs is sequestered in an extensive structure, predicted to prevent translation initiation. Nevertheless, 30S subunits do access the *tisB* TIR to initiate translation on the processed *tisAB* mRNA (+42). Thus, the structural feature that correlates with translatability is the long unstructured 5' tail present in the active *tisAB* mRNA but absent in the two inactive mRNAs. This is the same region that IstR-1 targets to sequester it in +42 *tisAB* mRNA, almost mimicking the structure in the +1 *tisAB* mRNA, and thereby rendering it inactive again. Therefore, the 5' end of the active *tisAB* mRNA must contain an element responsible for activation (Figure 9).

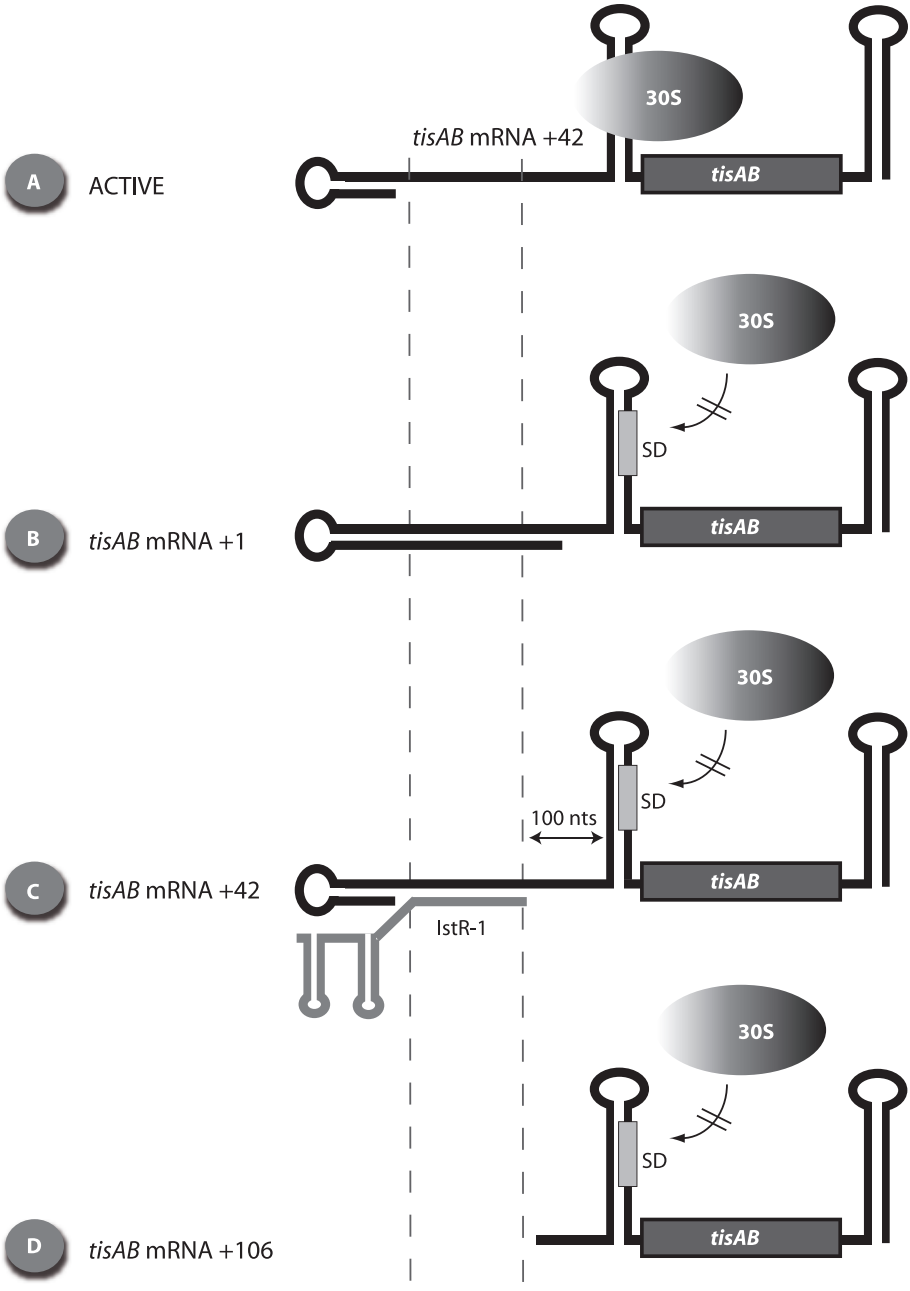


Figure 9. The *tisAB* mRNA leader determines the translation efficiency. The 5' end of the processed *tisAB* mRNA must contain an element responsible for translation – this region is indicated by the dashed lines. The structural differences in the 5' end of the transcripts are illustrated.

A ribosome standby site?

Since the two models described above were ruled out by our experiments, and the single-stranded region appeared to be the key to translation and regulation, we considered alternative explanations.

In the context of phage gene expression, de Smit and van Duin studied RNA folding kinetics, especially for structured RBSs (151-153). For initiation, ribosomes compete against structures in the RBS. If a structure is moderately unstable, this competition is won by the ribosomes, but very stable structures should efficiently prevent ribosomes from binding to an RBS (151). de Smit and van Duin studied the MS2 phage coat protein gene which is sequestered in a stable structure but, paradoxically, is still efficiently translated. How is this possible? They calculated that the time window during which the SD was accessible, i.e. the lifetime of the unfolded hairpin, was about 0.1 μs. For diffusing ribosomes at an estimated concentration of 8.5 μM in the cells, a time-window of about 4 ms would be required to obtain efficient ribosome binding. This discrepancy suggested the need for a different solution (142). If the ribosome were already on the mRNA, sitting on standby on a so-called ribosome standby site (a sequence non-specific ribosome loading site) in the vicinity of the RBS, diffusion would no longer be limiting since the local ribosome concentration would be extremely high. A ribosome could then relocate to the RBS when it transiently enters its open state; ribosome entry becomes proportional to the opening/closing equilibrium of the stem. Thus, the presence of a postulated ribosome standby site in the message for the MS2 coat protein gene could account for the observed in vivo efficiency of translation (142).

If we now go back to *tisAB* regulation – we propose that the unstructured region in the active *tisAB* mRNA serves as a ribosome standby site. Hüttenhofer et al (154) has shown that 30S ribosomes make sequence-independent contacts with 35-50 nucleotides. In the active *tisAB* mRNA about 15 nucleotides are entirely single-stranded, and the upstream structure is weak, generating a site accessible for 30S. The ribosomal subunit could then shift into place as soon as the *tisB* RBS structure opens.

Based on this concept, it is easy to envision how IstR-1 inhibits translation even though its binding site is far upstream of the *tisB* TIR; IstR-1 blocks the ribosome standby site, thereby preventing the initial ribosomal contact with the message. Based on van Duin and de Smit's studies, a standby site should be needed on the *tisAB* mRNA. The *tisB* SD is sequestered in a structure and, based on thermodynamic calculations, the ribosome should not be able to bind to the SD. The introduction of a ribosome standby site would solve this problem.

Standby required to overcome RNA structure

To ask whether the *tisB* SD stem is indeed inhibitory, I introduced base pair mutations in the strand opposite the SD sequence to weaken the structure. Structural mapping confirmed that the stem was destabilized and the surrounding structure unaffected. Translation assays and toe-print experiments showed that opening of the SD stem increased translatability of the two previously inactive *tisAB* mRNAs. Another striking observation was that the active +42 mRNA did not become more translation-competent with a weakened SD stem but was unaffected. This implies that the stable *tisB* RBS structure is not inhibitory for translation efficiency once the standby site is present on the mRNA.

By using an oligodeoxyribonucleotide that base paired between the standby site and the start of *tisB* translation initiation was inhibited. Thus a road block was created, which might suggest lateral sliding of the ribosomes. Free ribosome sliding on mRNAs has been documented before and could apply to *tisAB* mRNA (155-157). More direct biochemical evidence for ribosomes on standby was difficult to obtain. Sometimes a weak fMet-independent toe-print was visible in the standby region, which might represent a trace of standby ribosomes (unpublished). Efforts to cross-link ribosomes on standby were unsuccessful. In 2006, a study presented biochemical support for standby binding on artificial model RNAs (158,159); fluorescence resonance energy transfer (FRET) experiments were employed to study the ability of 30S binding to RNAs of increasing stability, exhibiting SD sequences of varying lengths. A rapid and SD-independent step was detected, completely dependent on a stretch a single-stranded nucleotides preceding an inhibitory structure.

Summary

The ribosome has two competitions to win to be able to initiate translation on *tisAB* mRNA; first it needs to compete against IstR-1 for the standby site, then it needs to compete against the internal structure for the SD. IstR-1 is the first described sRNA that competes against ribosomes on standby, rather than directly against initiating ribosomes. See Figure 10 for the regulation of *tisB* expression.

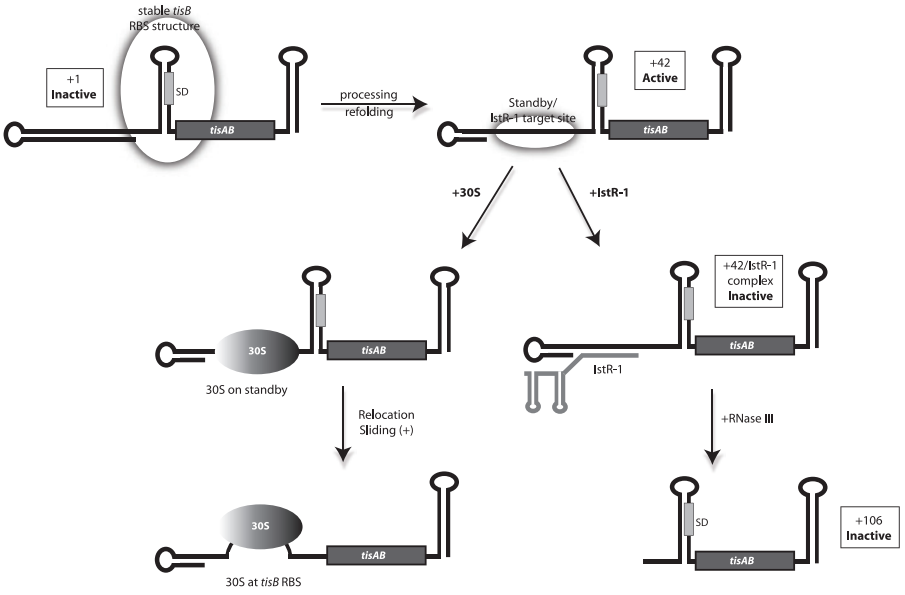


Figure 10. Model of regulation. In the model the sRNA IstR-1 competes with ribosomes on standby

Characterization of TisB (Paper II)

TisB is irreversibly toxic when over-expressed

Following our mechanistic studies of the IstR-TisB system, I turned to the nature of the toxic effect. We wanted to address the mechanism of TisB-dependent toxicity and therefore used overexpression conditions.

Upon plasmid-borne expression of *tisB* from an active (+42) gene construct I detected a growth arrest and a drop in viable counts. The effect was less severe and delayed when the +1 mRNA constructs were induced. We infer that this is due to the requirement for processing to generate the +42 mRNA. Induction of the RNase III-cleaved mRNA (+106) had no toxic effect, as expected. Under overexpression conditions, the TisB-mediated growth effects were irreversible.

Mode of action of TisB

Next we wanted to investigate the mode of action of the TisB toxin. By employing both localization studies, measurements of intracellular ATP levels and macromolecular synthesis rates, we could get a handle on TisB-mediated toxicity.

TisB is specifically localized to the inner membrane (IM), see Figure 11B. A reduction in replication, transcription and translation rates could be observed upon TisB expression. A severe RNA decay was also noticed under those conditions. Since we further detected a decrease in the intracellular ATP levels (Figure 11A), a probable mode of action of TisB was suggested as follows.

Once TisB is induced, it is targeted against the inner membrane. This insertion of TisB affects membrane integrity and abolishes the proton motif force. This in turn leads to a decreased intracellular ATP concentration and, as a consequence, lower transcription, translation and replication rates. An RNA decay may be attributed to the disassembly of ribosomes. Eventually, this leads to cell death and formation of ghost cells. Ghost cells are dead cells depleted of cellular contents, which are also generated upon Hok expression (125).

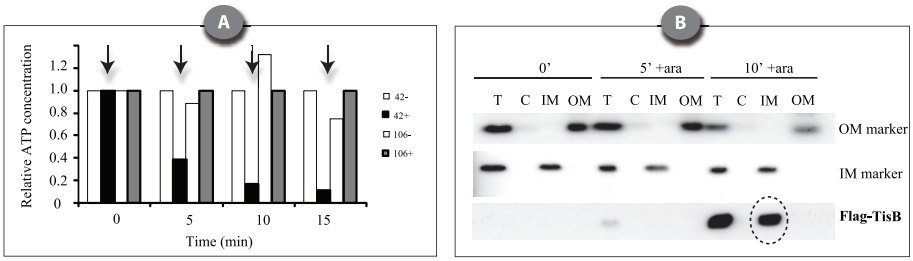


Figure 11. TisB-mediated effects on cells. A) TisB causes a drop in ATP levels. A luciferase assay was employed to measure the relative intracellular levels of ATP. The black bars represent TisB expression after arabinose (ara) addition from +42 constructs, highlighted by the arrows. B) Western blot experiments after membrane protein fractionation. T: total protein, C: cytoplasmic fraction, IM: inner membrane fraction, OM: outer membrane fraction. Inner and outer membrane markers were used, as well as a Flag-antibody to detect TisB. TisB localization is highlighted by a dotted circle.

Physiological role of the IstR1-TisB system

This scenario which results in cell death is only obtained as a consequence of overexpressing TisB. When chromosomal (single gene dosage) *tisB* is expressed, cell death is not observed. In order to detect minor growth differences, a competition experiment was conducted for a wt versus an *istR-1* deletion strain in the presence or absence of mitomycin C (an inducer of the SOS response). Mitomycin C treatment resulted in a growth disadvantage for the *istR-1* deletion strain compared to the wild type strain, indicating a much milder TisB effect at the single gene level.

Deleting the entire TA locus did not generate any detectable growth effect. The TisB effect could have been "diluted" in the redundancy of other SOS-involved TA systems present in *E. coli*, but also a triple deletion strain

for the so far identified SOS-related TA loci failed to generate a fitness phenotype. A recent study in which five of the most extensively studied TA loci were deleted could not demonstrate any growth phenotype either (160).

The lack of phenotype could also be attributed to the setup of experiments. All of the above experiments were conducted in bulk cultures. Thus, if there would be stochastic variation on the single-cell level it would have been overlooked. Possibly, only a small fraction of cells in the population may show a fitness phenotype. A recent study showed a TisB-dependence in persister formation during the SOS response (161). Persisters are dormant cells that display multidrug tolerance, and they only constitute a small fraction of a large population. Since TisB is synthesized at low levels, stochastic variation is expected, and the cells that achieve a high level of TisB would become persisters. Other toxins have also been implicated in persister formation, such as RelE and YafQ (162).

Summary

TisB is toxic when overexpressed by disrupting membrane integrity, ultimately causing cell death. This is however not the effect of single-gene dosage of TisB. TisB is specifically targeted to the inner membrane when induced during SOS conditions, which may lead to stasis. Since no growth advantage was observed for a wild type strain versus an *istR-tisB* deletion strain, TisB is probably induced in small amounts resulting in cell-to-cell variations.

Further studies

The terminator stem in *tisAB* mRNA is a stability determinant

When we made our plasmid constructions for the study above, we encountered a problem in the cloning procedures. We repeatedly obtained point mutations in the terminator stem for the active (+42) *tisAB* mRNA. This could have been coincidental or imply that the terminator stem is an important stability factor. In order to study the relationship between the stability of the mRNA and the translation of TisB, we *in vitro* transcribed +42 *tisAB* mRNA with and without the terminator stem to be used as templates in two different *in vitro* translation assays (Figure 12). One assay was derived from an *E. coli* extract, i.e. it did not only contain all the components necessary for translation but also RNases and other factors involved in decay of RNAs (*E. coli* S30 extract). The other assay was based on purified translation components, and thus lacks degrading activity ("PURE system"). Labeled methionine was included in the extracts for

detection of TisB. These assays clearly demonstrated that the presence of the terminator stem was important for stability of the transcript (Figure 12). In the S30 extract, almost no TisB was translated from the mRNA lacking the terminator compared to the cognate wt mRNA, whereas in the PURE system, differences in TisB levels were minor.

The introduction of point mutations in the terminator weakens the terminator stem, and thus rendering *tisAB* mRNA less stable. These data imply the terminator stem as a stability determinant, indirectly affecting toxicity levels.

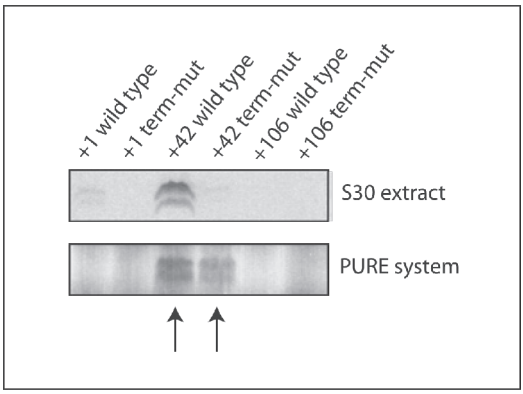


Figure 12. In vitro translation assays showing *tisAB* terminator stem as a determinant for mRNA stability. Synthesized *tisAB* mRNA species with (wt) or without (term-mut) the terminator stem were subjected to translation assays containing either RNases (S30 extract) or only purified components (PURE system). The intensity of detected bands correlate to the relative TisB translation.

IstR-2 function

The second sRNA, IstR-2, encoded in the *istR-tisAB* locus is under SOS control. To get a handle on the induction pattern under SOS conditions, mitomycin C was added to a wild type culture in midlog phase. Total RNA was extracted at different time points and IstR was assessed by Northern blot. Induction of IstR-2 was relatively fast, with increasing levels already after 10 minutes of induction, whereas IstR-1 levels remained constant. In order to ask directly whether IstR-2 also could affect TisB regulation, *in vitro* translation assays were employed. The results showed poor IstR-2 inhibition of TisB translation; IstR-2 was 30x less efficient than IstR-1. This was also confirmed by gel shift experiments conducted by Fabien Darfeuille, which showed weak IstR-2-*tisAB* mRNA binding. Chemical probing of IstR-2 and IstR-1 suggested a structural reason for the difference in effect on *tisAB* mRNA. Whereas the region of complementarity to *tisAB* mRNA is located in the unstructured 5' tail in IstR-1, this sequence is partially sequestered in a stem and thus not that accessible in IstR-2 (F.D., unpublished). We therefore concluded that IstR-2 might have other targets that needed to be under SOS control.

To identify potential IstR-2 targets, I used microarray studies. Samples were extracted from a designed arabinose tolerant *istR-tisAB* deletion strain,

containing either an arabinose-inducible control plasmid (163), an IstR-1 plasmid, or an IstR-2 plasmid prior to or after induction of the sRNAs. The results were inconclusive. In a new approach, an IstR-2 promoter deletion strain has been constructed, which would provide IstR-2 specific affects under the SOS response (single gene dosage of IstR-2). Samples prior to and after IstR-2 induction have been extracted and will be subjected to deep sequencing in a further attempt to identify IstR-2 targets.

MicF targets its own regulator Lrp, providing a positive feedback loop (Paper III)

Lrp: a putative MicF target

Many of the characterized sRNAs regulate multiple mRNA targets to generate a coordinated response to a specific environmental signal. In order to identify additional mRNA targets for the sRNA MicF (other than *ompF* mRNA (7)) an in-house developed computer algorithm was used which suggested *lrp* mRNA as a putative target (Reimegård et al, unpublished).

Lrp is a leucine-responsive global transcription factor that controls up to 10% of all genes (164-166). Its regulatory function may be affected by binding to leucine. Leucine may promote, reverse, or have no effect on Lrp activity (167-169). Lrp is a feast/famine regulator that controls genes involved in metabolic pathways in response to nutrient availability (especially amino acids and nitrogen bases), including its own gene, *lrp* (170). Lrp plays a central role in adaptations from nutrient-poor to nutrient-rich conditions, and vice versa (167). This implies that regulation of Lrp is expected to have a major impact on gene expression patterns (166). Interestingly, Lrp also negatively regulates *micF* expression (171).

MicF targeting of *lrp* is sequence-specific and Hfq-dependent

An earlier study showed that deletion of *lrp* resulted in a twofold increase in MicF levels (171). Conversely, we wanted to investigate whether the absence of MicF had any effect on Lrp levels. A *micF* deletion strain displayed a modest but consistent and significant increase (25%) in Lrp levels compared to an isogenic wild type strain. This suggested a MicF-dependent downregulation of Lrp.

Based on bioinformatic prediction, MicF should base pair to the *lrp* TIR, thereby occluding ribosomes from initiating. To validate this interaction, a plasmid reporter system was used (172). The 5' UTR of *lrp* mRNA was fused to GFP as a translational fusion on a moderate copy number plasmid. MicF was expressed from a high copy plasmid (172). When the MicF

plasmid was present the Lrp signal was decreased fivefold compared to the control plasmid. A point mutation introduced in the 5' tail region of *micF,* and the corresponding compensatory mutation in *lrp,* showed that *lrp* mRNA is a direct target of MicF.

In vitro experiments further validated MicF regulation of *lrp*. *In vitro* translation assays showed that MicF inhibits translation of *lrp*, and that this interaction is sequence-specific and Hfq-dependent. Toe-print analyses also confirmed those results (149), specifically demonstrating that translation initiation complexes could not be formed when MicF was present. All these results indicate that MicF base pairing to the *lrp* TIR prevents translation initiation.

MicF affects Lrp-controlled downstream target genes

When regulating a TF, its downstream regulated genes should also be affected. In order to study the effect on both Lrp-regulated genes (including *lrp*) and the Lrp protein level, a flag-tagged *lrp* strain was used

One well-studied Lrp target is the negatively regulated *livJ*, important for branched amino-acid transport (173). *livJ* was highly upregulated (about 20 fold, data not shown) in an *lrp* deletion strain. When pMicF was present in a wild type strain, *livJ* expression was highly increased compared to the control strain, reflecting the lower levels of Lrp available. This effect was abolished when the mutant pMicF was introduced. This clearly demonstrates that the MicF-mediated down regulation of Lrp is indeed reflected in changes of mRNAs for Lrp-regulated genes.

The effect of plasmid-borne MicF on the *lrp* mRNA level was only modest (< 2 fold downregulation), whereas the effect on Lrp protein level was more substantial (3-5 fold downregulation). This could reflect the autoregulation of Lrp (Lrp negatively reguates *lrp*). A decrease in Lrp protein levels lead to a derepression of *lrp*, resulting in an increase in *lrp* mRNA levels.

A positive feedback loop

Lrp is known as a negative regulator of MicF (171), and here we demonstrated MicF to be a negative regulator of *lrp* expression. Thus, this regulatory pattern constitutes a positive feedback loop (174).

To validate this more directly, we employed a mutant MicF plasmid, where mutations were introduced in *micF* located such that they do not affect the *lrp* interaction. This mutant MicF was still active in Lrp regulation but distinguishable from the chromosomally encoded MicF by employing specific matching DNA probes on Northern blots. IPTG was added to induce MicF-mutant. Together with MicF-mutant accumulation, the chromosomal *micF* expression level was substantially increased. The chromosomal MicF

increase was dependent on Lrp, since the MicF levels were unaffected in an *lrp* deletion strain. These experiments demonstrate MicF as an indirect positive regulator of its own synthesis, through a positive feedback loop including Lrp.

This positive feedback loop may be especially important when fast increases in expression levels are required, to promote rapid adaptations in response to changing environmental conditions. MicF could then be considered as a first player in this circuit to generate a fast change in Lrp levels, affecting the Lrp regulon. Another sRNA, ArcZ, has also recently been shown to be part of a positive feedback loop with the regulators ArcA/ArcB (69), and the sRNA RyhB and Fur also displays such properties (175).

Mixed feed-forward loop?

Another interesting aspect regarding MicF-Lrp regulation is that both MicF and potentially Lrp regulate *ompF* porin expression. One report demonstrated the Lrp-dependent regulation of *ompF* as indirect via MicF (171), but a recent study detected an interaction between Lrp and the *ompF* promoter (166), indicating a direct regulation. If true, Lrp positively regulates *ompF* expression at the transcriptional level and MicF inhibits *ompF* expression post-transcriptionally. A mixed regulatory pattern as such, where two modes of regulation occur on a single target, is described in a study by Shimoni et al (143). The type of feed-forward loop where one regulation is positive and one is negative is referred to as an incoherent circuit (143,176). This mixed feed-forward loop consisting of Lrp, MicF and *ompF* is even more complicated than depicted in this paper, since *lrp* is also autoregulated at the transcriptional level and, in addition, regulated by MicF (Figure 13).

The biological significance of such a complicated circuit may be the following. Once Lrp levels are high (in e.g. in nutrient poor media (169)) repression of MicF will allow for even higher *lrp* expression, as well as increased *ompF* expression. Conversely, high expression levels of MicF (e.g. in nutrient rich media or under osmotic stress (177)) lead to a repression of Lrp levels, promoting an increased MicF level to ensure *ompF* expression is inhibited. Thus, an incoherent feed-forward loop accelerates the response time. Since Lrp also regulates its own transcription, it ensures a certain steady state level with a built-in buffering system.

Summary

MicF downregulates its own repressor, Lrp, thus providing a positive feedback loop. Such loops ensure fast adaptations to changes in the

environment. MicF can therefore be considered as the first actor in the Lrp regulon.

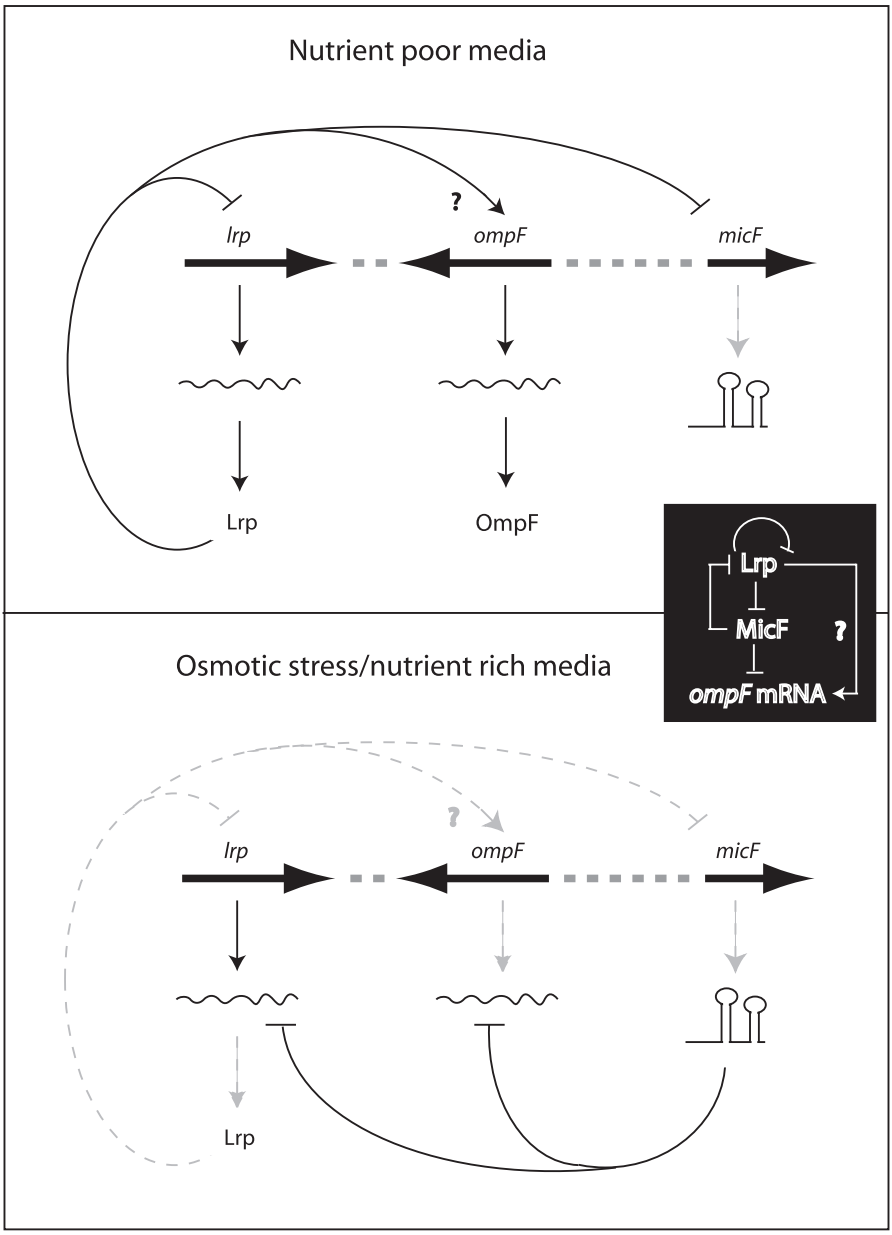


Figure 13. A model of the regulatory outputs of the Lrp-MicF autoregulation under different conditions

Discussion

The aim of my investigations was to study sRNA-mediated regulation in *E. coli*, in the hope to gain a broader view of gene regulation and translational regulation in general, since small non-coding RNAs are ubiquitous in all kingdoms of life. Here I have presented results and conclusions from two different sRNA-target mRNA interactions, including one toxin-antitoxin locus. In this section I have tried to look at sRNA-regulation from a broader perspective, with emphasis on translational control. In addition, specific issues related to my work are covered.

Benefits of using regulatory RNA

Non-coding RNAs have been implicated in various regulatory functions and have important roles in both prokaryotes and eukaryotes. Since the abundant bacterial sRNAs act stochiometrically, they tightly repress their target genes when present in excess, but have little or no effect when in shortage. Dynamic simulations have demonstrated a threshold below which the sRNA no longer efficiently represses its target and becomes sensitive to intrinsic noise (such as transcriptional bursting) (48). Under those conditions, sRNAs are not well suited to repress targets since they, unlike the TFs, are consumed together with their targets.

It can be beneficial for bacteria to be exposed to high noise levels, to promote an increase in the phenotypic diversity under certain conditions. A disrupted fidelity due to high noise levels may however be harmful. Thus, sRNA or TF-based regulation may be advantageous under different conditions.

A stress response, such as osmotic stress (a strong environmental cue), requires a fast regulatory response and here sRNA-regulation is advantageous, since it ensures fast adaptations for the cells. Dynamic simulations have demonstrated a faster response for sRNA-regulation compared to TF-regulation (143).

These rapidly-acting regulators may act on several target genes and may prioritize their usage of different mRNA targets, allowing them to control entire physiological networks (44,145). This prioritization ability may depend on different binding rates between the sRNA and the targets. Some sRNAs have been shown to bind to many mRNA targets within the same

network, e.g. RyhB involved in iron metabolism (39,178). It is not possible for a single sRNA to control as many genes as a TF does, since the pool of sRNAs must exceed the combined pool of targets for efficient regulation without any leakage/noise (144). Another possibility to control a complete physiological network is to target a sigma factor or TF. The sigma factor σ^S is extensively regulated by sRNAs (70). Several TFs are also sRNA-controlled, including Lrp identified in this study (Paper III). Even though the regulatory effect on the TF may be small, all the genes regulated by the TF may be affected to different extents. In Paper III we showed that MicF downregulation of *lrp* expression leads to a derepression of the Lrp-controlled gene *livJ*.

sRNAs are also predicted to be cost-effective for the cell, since they are small in size and do not need the extra step of translation. The metabolic cost may differ dependent on the condition (this is also valid for protein-based regulation) (145).

By applying different combinations of regulations to control gene expression, tight regulation may be accomplished under every desired condition. Mixed regulations with so-called feed-forward loops, when two different levels of regulations act on the same target, may be superior under many conditions (143). Small RNAs are now established as relevant regulators for gene control.

Non-coding RNAs in all kingdoms of life

Regulatory ncRNAs have been found in all kingdoms of life. In bacteria they are primarily involved in stress adaptations (54), and in plants and metazoans they play important roles in developmental timing and cell differentiation (179,180). In eukaryotes, miRNAs play an important role in regulating gene expression involved in vital functions. New classes of regulatory sRNAs are constantly being identified, demonstrating the importance of riboregulators. miRNAs are usually not co-degraded with their targets when exhibiting a relaxed target complementarity, enabling them to regulate targets over again, in contrast to their bacterial counterparts (181). Thus, in some aspects they can almost be considered as comparable to TFs. Many more proteins are involved in miRNA-dependent than in sRNA-mediated regulations. Approximately 60% of all protein-coding genes in mammals have been predicted to be controlled by miRNAs (182). Should we expect a similar fraction of genes to be controlled by sRNAs in bacteria? At present 2% of the *E. coli* genes are known to code for sRNAs – is this close to saturation? Since sRNAs often have multiple targets, 2% may account for a much higher percentage of controlled genes. Small RNAs that are not consumed together with the mRNA target may be identified, similar to miRNA-mediated regulations. We may also find sRNAs with ribozyme

activity in the future, as is already known for cis-encoded (riboswitch) elements (1,2). In some bacteria no Hfq homolog has been found (88). Potentially these bacteria can perform riboregulation without the help of Hfq, or may utilize some other helper component.

It has recently been shown that riboswitches in *Listeria monocytogenes* can have dual functions: they act both as a riboswitch in cis, and as an sRNA in trans regulating other targets (183). If this applies to more than the two riboswitches studied in that paper, a new distinct class of regulatory RNAs may emerge in bacteria. The dual function of riboswitches could of course be either species dependent or more universal.

The analogous functions of bacterial sRNAs to eukaryotic miRNAs have been known for a while, but now we also have an additional bacterial RNA-based system which is functionally analogous to RNAi; CRISPR (105). The CRISPR research is still in its infancy, and certainly provides challenges for the future.

This study has only considered *E. coli* laboratory strains, but sRNAs are of course also present in pathogenic bacteria, playing important roles for e.g. virulence (184-188), and results obtained in harmless bacterial species can often be extrapolated to similar pathogenic species. A study in *Salmonella* showed that virulence was severely affected in an Hfq-deletion, indicating the importance of sRNA-mediated regulation in virulence (98). Hfq, as a virulence factor has also been reported for several other pathogenic bacterial species (189-192). Some sRNAs, such as RNAIII in *Staphylococcus aureus* and Qrr1-4 in *Vibrio cholerae*, act as coordinators of pathogenicity.

Localization of sRNAs

One interesting question is where regulatory RNAs are localized. In eukaryotes they may have sub-cellular locations depending on their biological function. Eukaryotic regulatory RNAs have recently been found in P-bodies (discrete cytoplasmic loci) (193). P-bodies may act both as "decay-centers" and/or storage sites for miRNA-repressed mRNAs. In the latter case, the repressed mRNAs may later be released into the actively translating ribosome fraction in a cost-effective manner (194).

In bacteria, RNase E and Hfq have been implied to be localized to the membrane (11,195,196). Does this mean we could expect sRNAs to also be localized in a similar manner, since most of them are Hfq-dependent? It has been shown that *ptsG* mRNA must be targeted to the membrane for the sRNA SgrS to efficiently control its expression (197).

A recently published article in Nature (198) addressed mRNA localization and diffusion patterns in bacterial cells. This elegant study demonstrated that full-length translating mRNAs stay close to their sites of transcription, rather than diffusing away, implying a spatial restriction and dedicated

compartmentalization in bacteria (*E. coli* and *Caulobacter cresentus*). They studied six different mRNAs with high-resolution microscopy. All six mRNAs were co-localized with their genes. Not only did transcription and translation occur at a dedicated place, but also RNase E co-localized with the genes in question. This implies that bacteria have functional compartmentalization for RNA processes, even though they lack internal organelles. The authors suggest that the use of the chromosome as a spatial organizer may explain the observed gene clustering. Since many genes coding for interacting proteins cluster together, a limited mRNA diffusion, together with a defined place for translation, might aid rapid interactions. These findings will have implications for sRNA-mediated regulations.

The diffusion patterns of sRNAs after transcription are not known. Do they stay localized at their genes or will they rapidly spread throughout the cell? Small RNAs are seldom encoded next to their target genes – should we expect a more uniform dispersion of them in the cells compared to the "average mRNA"? If mRNAs are immobile, sRNAs must get localized to their target sites within a relatively short time window, since they must generate fast responses. It is difficult to envision how this would work since Hfq is essential for most sRNA-target interaction, and Hfq has recently been reported to be localized near the membrane. Note that Hfq is required only for trans-encoded sRNAs, where it has essential roles in e.g. stabilizing the RNAs. This implies that sRNAs need to rapidly find Hfq after their transcription is completed. Plasmid studies have estimated mRNA diffusion rates to be 0.3-0.03 $\mu m^2 s^{-1}$ (199,200), but it is not certain that the same diffusion rates apply to sRNAs, nor do we know whether they diffuse freely. It is also possible that they require assistance in this localization by some unknown factor. These are all important questions that need to be solved to obtain a deeper understanding about gene regulation in bacteria.

It would also be of great interest to study the diffusion patterns of some inactive, stable primary transcript, like *tisAB* mRNA. Since there is a pool of stable, translationally inert *tisAB* mRNA, it could either be dispersed throughout the cell or stay localized at its transcription sites due to e.g. limited diffusion. Available data suggests a localization even for *tisAB* mRNA since its interaction partner IstR-1 is encoded next to it, i.e. if this clustering hypothesis is correct.

Additional characteristics of riboregulation

Our view of how riboregulation works has changed over the years. Some mechanisms seemed unlikely or were simply not known earlier, such as the preferential use of riboregulation in cis, sRNA-targeting in coding regions, long antisense transcripts, and uncoupled transcription-translation.

In gram positive bacteria, such as *Bacillus subtilis*, many riboswitches have been identified, where most of them work at the transcriptional level by transcription termination/attenuation, whereas in gram negative bacteria most of the riboswitches identified (not as many as in gram+ species) control translation (134). Why does translational control mainly apply to gram negative bacteria? One hypothesis is that gram positive species have more genes clustered in long operons. Thus, they would benefit from transcriptionally regulating their operons before they are transcribed to full-length transcripts in order to save energy (44). It is also interesting to note the different usage of riboregulators for the same loci in different species. The *glmUS* mRNA is regulated by a cis-acting riboswitch in *Bacillus subtilis* (201) and by trans-acting sRNAs in *E. coli* (28).

To control gene expression by targeting translation is important since this is the last opportunity to prevent a protein from being synthesized. Translation initiation, being the rate-limiting step, is often subjected to regulation by either internal structures (riboswitches or inhibitory structures) or external elements, such as sRNAs (44). It is therefore intriguing that the regulatory RNA MicC binds in the coding region of an actively translating mRNA, to promote rapid RNA decay (66). Prior to this study this was unanticipated, since it was expected to be difficult for an sRNA to access a region of an mRNA that was actively been translated.

In 2007, a study was published in which an sRNA, SR1, in *Bacillus subtilis* base paired downstream of the *ahrC* SD in the coding region to induce structural changes, rendering the message inactive, thereby indirectly inhibiting translation initiation (202).

An intriguing recent and anticipated finding is the presence of long antisense (as) transcripts in various bacteria (40,41,86,203), which are also known from many other organisms (204,205). The bacterial as-transcripts were identified through deep-sequencing (40,41) and/or Hfq-binding properties (86). The described Hfq-binding sites within these RNAs seem to be enriched in the region opposite the TIR in the sense mRNA (86). It is not known whether all of the as-RNAs are functional and/or whether they require Hfq for potential regulation. The regulatory effect of a 1.2 kb as-transcript on its cis-target in *Salmonella enterica* was recently reported (87). These long cis-encoded RNAs in bacteria may represent an over-looked new class of regulatory RNAs. Alternatively, transcription of the as-gene may prevent transcription of the sense gene by so-called transcriptional interference. If so, it is the transcription process as such that is important (206-209). These enigmatic as-transcripts now await characterization.

The *tisAB* mRNA is subjected to three different inhibitory structures; first the primary mRNA employs internal sequestration of the ribosome standby site to render it inactive, second, an internal structure blocks the TIR to prevent translation initiation and, third, IstR-1 blocks the ribosome standby site to inhibit ribosomes from binding to the message. This is an example

where translation is prevented by only employing different types of inhibitory RNA structures. Since bacteria have coupled transcription-translation, an mRNA not being translated during transcription is usually rapidly degraded in the absence of protecting ribosomes. This is not the case for the +1 *tisAB* mRNA – it is stable with a half-life of ≈ 15 minutes (unpublished). This transcript is dependent on a slow processing of the 5' end to generate a translation-competent transcript, ready to be either targeted by 30S or IstR-1 depending on the conditions. (72)

Also the stable *hok* mRNA has uncoupled transcription-translation. Slow 3' end trimming renders this transcript active, which may be either translated or inactivated, depending on absence or presence of Sok (123,124). This TA system is similar to the IstR1-TisB system in many different aspects, considering both the complexity on the RNA level and the characteristics of the toxins (84). Whereas the Hok-Sok system works due to differences in RNA stabilities, the IstR1-TisB system relies on the molar ratio of the RNAs involved (the *tisAB* mRNA pool needs to exceed the IstR-1 pool to be translated), and probably on stochastic variations.

Standby requirement: a common theme?

A standby site on an mRNA as a means to improve translation efficiency may be a more general feature than earlier envisioned. Many TIRs in mRNAs may exhibit structures that could impair translation initiation. The presence of a sequence non-specific ribosome loading site on the message would then be advantageous. Our data present indirect evidence for a ribosome loading site/standby site, in line with the proposal by de Smit and van Duin (142).

What then is a standby site? Does it have any structural features or necessary signatures? This is something we can only speculate about. There is some biochemical support for a ribosome loading site as being unstructured (159). Our study also supports lack of structure as the major determinant.

Many operons encoding ribosomal proteins are autocontrolled using one of the encoded proteins as a translational repressor (210). Entrapment models have been proposed for the control of ribosomal proteins S4 (211) and S15 (212), with their respective mRNAs. The 30S is entrapped in an inactive complex due to S15/S4 binding to a pseudoknot present in the *rpsO* mRNA/α-operon. This implies that 30S can stably associate, but does not form an active complex in presence of the repressor. In 2007, an elegant study by Marzi et al (213) investigated autoregulation of *rpsO* mRNA by S15. Cryo-electron microscopic experiments were conducting on different stages of the *rpsO* mRNA-S15 complex during the translation initiation pathway. 30S was trapped at the SD sequence but in a confirmation that

made all necessary contacts to initiate translation impossible. Upon S15 dissociation, 30S was able to be accommodated to form an active IC. This entrapment may be considered a variant of ribosome standby, where the 30S is already in place. Marzi et al (213) speculate that there is a ribosomal platform binding center that may be responsible for binding structured RNA elements.

We tried to map the ribosome standby site in *tisAB* mRNA but failed. Now we have turned to another approach in our lab. By designing stretches of scrambled combinations of nucleotides in front of the MS2 RBS-containing structure fused to GFP (as a read out), also including the different *tisAB* mRNA species, we hope to detect any features correlated to standby properties (Sterk, unpublished).

It would be interesting to study the stability of the RBS-containing regions in many mRNAs to assess the occurrence of inhibitory RNA structures, in order to predict the likelihood of requiring standby sites in the vicinity. If a structure is too stable to be helped by standby, it may need to be physically broken by e.g. translational coupling. This was tested in our lab a few years ago by designing different translational fusions of the very stable *repA* SD-stem to lacZ. Translational coupling is required in wild type scenarios to open this stem. This was also confirmed – introduction of a standby site was not enough to relieve this inhibitory stem (unpublished results). To get a handle of the RNA structural stability interval for standby requirements, systematic studies need to be conducted.

Standby binding may be advantageous for reasons other than dealing with stable structures. Three-dimensional searches for specific sites may constitute an efficiency problem for both ribosomes and DNA binding proteins (in presence of a large non-specific background). If we consider DNA binding proteins, they do not diffuse randomly in the cell to eventually find rare recognition sites on the DNA. Non-specific binding between a TF and the DNA renders diffusion along the DNA possible, generating faster binding rates, thereby improving efficiency. It has been shown that the Lac repressor spends most of it time (~ 90%) non-specifically bound to the DNA searching for its recognition sites (214). This should be relevant for the 30S as well. By introducing ribosome standby sites on mRNAs (especially if the TIR is sequestered), the concentration of ribosomes already in place would increase, thus translation should theoretically be enhanced. Such standby sites could then be considered as rate-enhancing elements.

Many questions remain. How do ribosomal proteins make contacts with the RNA? Are there any structural/sequence features involved (mutating the *tisAB* standby site did not have any effect)? What is the RNA stability limit for a standby site to be functional as a translational enhancer element? We speculate that standby binding may be frequent in bacteria.

Relevance of chromosomally encoded TA systems?

In Paper II, I characterized the toxin, TisB, encoded by *tisAB*, and regulated by IstR-1. TisB is induced under SOS conditions, but there are many other stress conditions during which toxins in TA systems are induced. There is not only a redundancy among the type I TA involved in the same response, such as TisB-IstR1, SymE-SymR and yafN-yafO (25,83,215), but also among the same TA systems. There are four different copies of Rdl-Ldr and Sib-Ibs, as well as many Hok-Sok copies (114). Why this redundancy? It is not known when those toxins are induced, whereas the antitoxins are constitutively expressed. Interestingly, the only two trans-encoded type I TA modules (*istR-tisB* and *shoB-ohsC*) are also only present in one copy each (25,126). All the other type I TA loci are cis-encoded. There is also much redundancy among homologous type II TA systems (216,217).

The biological relevance of the plasmid-encoded TA systems is clear: they take part in e.g. plasmid maintenance. The stable toxins kill the cells in the absence of the unstable antitoxin, thus ensuring killing of plasmid-free cells (84). The biological relevance of the chromosomally encoded TA modules has remained enigmatic. TAs have been implied in various stress responses, but no phenotype has been observed in the absence of the TA loci (218). Not even a deletion strain lacking five of the most described TA systems displayed any difference in the growth phenotype compared to a wild type strain when exposed to different stress conditions (160). In our study (Paper II), we deleted the three identified SOS-related TA loci and conducted many fitness experiments, unsuccessfully. What would be the advantage of carrying so many TA loci (219)?

One possible explanation for the many type I TA loci present on the chromosome, which are also present on accessory elements, may be horizontal transfer. In that perspective these loci (e.g. *rdl-ldr*, *sib-ibs* and *hok-sok*) would be non-functional, thus the toxins would never be expressed, being merely "selfish DNA". This could apply to the chromosomal *hok-sok* loci since they seem to be mutationally inactivated (112).

There is a controversy whether MazF-MazE is a bacteriocidal or a bacteriostatic system. Gerdes and co-workers claim the toxic effect caused by MazF-induction to be reversible (117), whereas Engelberg-Kulka and co-workers observe non-reversible toxicity under their conditions used (122). The latter group recently demonstrated a population-dependence of MazF, due to a specific quorum-sensing molecule (220). This is an on-going debate.

The toxin MazF is important for development in *Mycococcus xanthus,* when the majority of cells undergoes cell lysis to promote spore formation of the remaining cells (221).

We speculated in our paper that TisB might be stochastically expressed due to fluctuations in gene expression and we would therefore not detect any TisB-related effects in bulk measurements. A recent report confirmed this.

They showed that TisB was the main determinant for persister formation during SOS conditions (161).

Persisters: dormant cells

Persister cells are dormant cells that exhibit multidrug tolerance and can survive harsh conditions. They are pre-existing phenotypic variants in cell populations that do not divide, thereby surviving prolonged antibiotic exposure. They spontaneously enter this stage in a stochastic manner, with an increasing accumulation in stationary phase. Only a few genes have been implicated in this phenotype switch. No significant phenotype has been observed for deletions in knock-out libraries, suggesting a high degree of redundancy. The dormant cells have been implicated in some chronic infections, such as e.g. cystic fibrosis and may have a main role in the recalcitrance to antibiotics. Even though such multidrug tolerant cells have been described decades ago, still not much is known about them. (222)

It has recently been demonstrated that many toxins are highly upregulated in persister cells, e.g. RelE, MazF and YafQ (217,223,224). This implies the involvement of many toxins in the formation of those cells. The TA redundancy could explain why no phenotype has been observed in knock-out libraries. The gene *hipA*, encoding another toxin, was the first gene to be linked to persister formation (225). HipA phosphorylates elongation factor Ef-Tu rendering it inactive, which inhibits global translation, suggesting a HipA-mediated mechanism for persister formation via cell stasis (226).

A recent publication demonstrated TisB as the major player in the formation of persister cells under SOS conditions (161). A *tisB* deletion displayed a severe decrease, and an *istR* deletion an increase, in the induction of persister cells. TisB, identified in our study as an inner membrane targeting protein, affects the proton motif force and ATP concentration to induce the formation of these dormant variants of cells.

What determines when TisB should be synthesized? Only a small fraction of the cells becomes dormant, indicating that TisB is not expressed in all cells, or at least not to a level that induces the switch. A very recent study has demonstrated the generation of cell-to-cell variations causing persister cells through threshold regulation (227). For stochasticity to matter, the ratio between IstR-1 and *tisAB* mRNA must be close to the threshold mentioned earlier (see figure 7). Once the intrinsic noise is high it will be reflected in a burst in TisB translation. The balance around this level of RNA molecules ensures fluctuations in *tisB* expression, thereby promoting persister formation. Small RNAs are not suitable as high-fidelity repressors around this threshold, but when fluctuation is wanted to obtain cell-to-cell variations, sRNA-regulation is advantageous. How many TisB proteins are required to make this cell switch is not known. To further study these

stochastic events, single cell experiments should be conducted. Such studies can be expected to give interesting and new insights.

Conclusions

The publications presented in this thesis show the following;

Paper I

I The inactive primary *tisAB* mRNA needs 5' trimming to become translation-competent
II No translational coupling or structural rearrangement occurs on *tisAB* mRNA to render it active (TisA is a never translated)
III *tisAB* mRNA contains a ribosomal standby site (reducing the stability of the RBS-containing stem increases translation efficiency)
IV IstR-1 competes with ribosomes on standby rather than initiating ribosomes

Paper II

I TisB is toxic when overexpressed
II The hydrophobic TisB is specifically targeted to the inner membrane which causes RNA degradation, a reduction in transcription, translation and replication rates, and a decrease in intracellular ATP levels
III A wt strain out-competed an Δ*istR1* strain, displaying a growth defect during SOS conditions
IV A competition experiment between an *istR-tisAB* deletion strain and its isogenic wt strain displayed no fitness effect

Paper III

I MicF specifically targets *lrp* mRNA in a Hfq-dependent manner to inhibit translation initiation
II MicF primarily inhibits translation of Lrp
III MicF and Lrp provide a positive feedback loop that affects the Lrp regulon.

Svensk sammanfattning

Våra arvsanlag, generna, består av DNA som är uppbyggt som en dubbeltrådig spiral. De allra flesta känner till DNA, men den här avhandlingen handlar om RNA, en systermolekyl till DNA. Vad är då RNA?

mRNA som ett intermediat mellan gener och proteiner

RNA har en mängd olika funktioner i cellen, varav en viktig funktion är att det fungerar som ett intermediat mellan gener (DNA) och deras produkter, proteinerna. Dessa RNA kallas budbärarRNA eller mRNA. När en gen är aktiv tillverkas mRNA, en enkeltrådig kopia av genen som fungerar som en mall för proteinsyntesen (**DNA → RNA → protein**). Proteiner är viktiga för cellers struktur och utför arbete. Varje protein bestäms av informationen som finns i dess gens kod. När vi behöver mer eller mindre av ett specifikt protein (beroende på yttre faktorer, såsom t.ex. mattillgång), måste den gen som kodar för det proteinet antingen slås på eller av. Proteinerna är de molekyler i cellen som är inblandade i det mesta; de kan fungera som enzymer, kan ha styrfunktioner, kan transportera syre (t.ex. hemoglobin), och vara antikroppar. Det är livsviktigt att rätt protein i rätt koncentration finns i cellerna vid alla tillfällen.

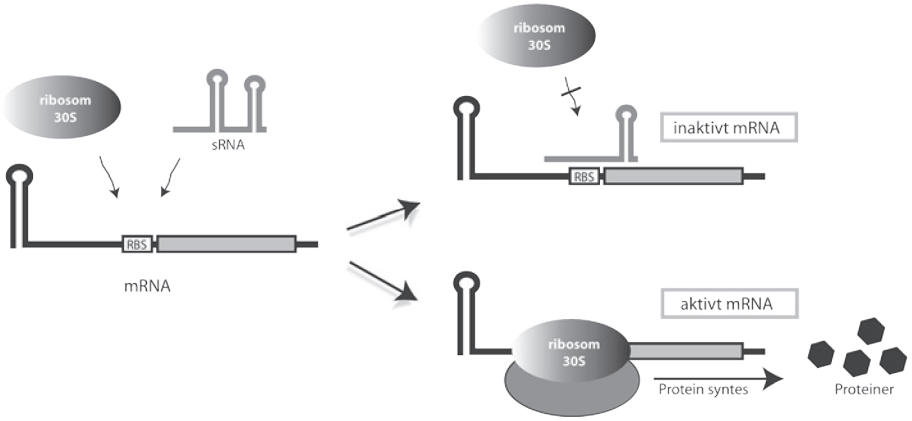
Geners aktivitet måste regleras

Genuttrycket måste vara flexibelt, d.v.s. genernas aktivitet måste regleras för att snabbt kunna anpassa proteinernas nivåer i olika situationer. Speciella styrproteiner (s.k. transkriptionsfaktorer) kan binda till genen och därigenom antingen påskynda eller motarbeta kopieringsmaskineriet. Detta kommer då att påverka hur mycket av proteinet som görs. På senare år har man även funnit att genaktiviteten kan styras på mRNA-nivå. Detta sker ofta med små regulatoriska RNA som kallas sRNA ("small RNAs"). Att ett litet RNA kan reglera ett mRNA beror på en speciell egenskap: RNA-molekyler är enkeltrådiga. DNA har två trådar bestående av kvävebaser (inklusive socker och fosfat) som är komplementära till varandra (kvävebaserna bildar par med varandra), vilket gör molekylen väldigt stabil och skyddad. Eftersom RNA enbart har en tråd kan en annan matchande tråd binda till den. Det är denna egenskap som gör att ett mRNA kan regleras av ett litet sRNA.

Beroende på var ett sådant sRNA binder minskar eller ökar proteinsyntesen från ett mRNA.

Ett sRNA kan inaktivera ett mRNA

Den fabrik som tillverkar protein utifrån mRNA-mallen heter ribosom. Ribosomer känner igen korta regioner (s.k. ribosombindningsställen: "RBS") i början av mallarna och fäster där för att påbörja syntesen. Det är just sådana ribosombindningsställen som sRNA oftast binder till för att förhindra att ribosomer kan binda. Vinner sRNA denna tävlan bildas inget protein – mRNA blir inaktivt och kan inte längre fungera som en mall (Figur 1). Ett inaktivt mRNA som inte längre är delaktig i proteinsyntesen blir dessutom lätt sönderklippt .



Figur 1. En illustration av hur ett mRNA kan inaktiveras av ett sRNA. 30S är den del av ribosomen som binder till ribosombindningsstället (RBS) på mRNA så att proteinsyntesen kan börja.

Avhandlingen

I denna avhandling har jag studerat sRNA i tarmbakterien *Escherichia coli*, vilket förhoppningsvis leder till en djupare förståelse för hur gener i allmänhet kan regleras på RNA-nivå. Vi vet att sådan reglering är universell och förekommer i alla organismer. Här följer en förenklad beskrivning av de delarbeten som jag inkluderat i min avhandling.

Studie 1

Vi studerade ett sRNA kallat IstR-1 som reglerar ett mRNA. Märkligt nog binder det inte till ribosombindningsstället utan långt därifrån. Ändå lyckas det att förhindra ribosomer från att påbörja sitt arbete. IstR-1 binder till en

region på mRNAt som också visade sig nödvändig för ribosomkontakten. Denna region behövs för just detta mRNA p.g.a. att det normala ribosombindningsstället där ribosominteraktionen brukar förekomma i detta fall är ”gömt” i en struktur. Då måste ribosomen istället tillfälligt binda till ett annat ställe och avvakta tills det riktiga ribosombindningsstället visar sig igen. Vi kallar denna region för ett ”ribosom-standby-ställe”. Om ribosombindningsstället på ett mRNA är otillgängligt kan proteinsyntesens effektivitet ökas avsevärt om ett ”ribosom-standby-ställe” finns nära.

Studie 2

I denna studie har vi karakteriserat proteinet som det mRNA i studien ovan kodar för. Det rör sig om ett toxin (ett gift för cellen) som produceras då cellerna utsätts för DNA-skador. Jag visade att detta toxin påverkar det inre membranet (en del av cellskalet), och detta i sin tur gör att energitillverkning upphör. Då stannar DNA-, RNA- och proteinsyntesen av, och cellerna slutar växa. Beroende på hur mycket toxin som produceras påverkas cellerna olika mycket. Vi tror att detta toxin är viktigt vid förekomsten av DNA-skador för att cellerna skall få tid att utföra de välbehövliga DNA-reparationsarbeten som måste utföras.

Studie 3

Vi har studerat ett sRNA, MicF, som reglerar syntesen av proteinet Lrp. Lrp kontrollerar gener som kodar för proteiner som behövs för bl.a. aminosyretillverkning och transport av små molekyler. En intressant aspekt här är att Lrp nedreglerar MicF-nivåer (genom att binda till *micF*-genens DNA), och i sin tur är nedreglerad av MicF (som binder till Lrp's mRNA). Detta leder till en positiv återkoppling (feedback loop) som gör att var regulator för sig kan öka sitt eget uttryck genom att trycka ner den andra under lämpliga betingelser.

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Within “Micro” there is one group of greater importance than others ☺ No, I am kidding, but a group of greater importance to me, that is my own group. I would like to thank both present and former group members. I have collaborated with Fabien (former post-doc), Erik and Johan and that has been very much fun, even though sometimes challenging (some people preferred to work mainly in the evenings...). The conferences have always been fun to attend, especially the evening sessions ☺ (the poster sessions usually ends in a bar). Erik has been my office-mate for some time now and also my only colleague still working with traditional sRNA-work (though we are now turning into a CRISPR-group). Johan, a special thanks to you for conducting many bioinformatical searches for me. Everyone: Nadja, Erik,

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