Contract no.:	**LSHG-CT-2005-018618**
Project acronym:	**BACRNAs**
Project title:	**Non-coding RNAs in bacterial pathogenicity**
Instrument:	**STREP**
Thematic priority:	**Life Science, Genomics and Biotechnology for Health**

FINAL ACTIVITY REPORT

Period covered: from 01.02.2006 to 31.01.2009

Start date of project: 01.02.2006

Duration: 36 months

Coordinated by:
Prof. Renée Schroeder, University of Vienna
Renee.schroeder@univie.ac.at
www.projects.mfpl.ac.at/bacrnas

supported by European Union's FP6
Life Science, Genomics and Biotechnology for Health
LSHM-CT-2005-018618

TABLE OF CONTENT

1 PROJECT EXECUTION

1.1 Project Objectives

"Non-coding RNAs in Bacterial Pathogenicity"

The BACRNAs team studied the role of non-coding RNAs (ncRNAs) in the regulation of bacterial virulence with the long-term objective to generate fundamental knowledge that can be directly applicable for therapeutic interventions to combat bacterial infections.

In bacteria, small **non-coding RNAs (ncRNAs)** are involved in the regulation of a growing number of adaptive processes like for example quorum sensing, transition to stationary phase, iron homeostasis, the SOS response, and bacterial virulence. Antisense RNAs, a class of ncRNAs that act by base-pairing to RNA targets, are responsible for the maintenance of many bacterial plasmids carrying antibiotic resistance genes. In the project performed by this consortium, we studied ncRNAs in **pathogenic bacteria** like *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes.* While studying the functions of ncRNAs, we are discovering new RNA regulatory networks that control bacterial pathogenesis and related mechanisms like bacterial stress and environmental adaptation.

Infectious diseases are the second-ranking cause of death worldwide. Yet, despite increasing incidence of bacterial resistance to existing drugs, antibiotics development and discovery in the pharmaceutical industry is declining. "*We need new approaches, beginning with the recognition that the antibiotic crisis of wealthy and poor countries are the same*" (quote by Carl Nathan, October 21st 2004, Nature). Many of the drugs used to treat bacterial infections target just a few classes of enzymes, briefly those involved in synthesis of proteins, nucleic acids, cell wall or folate. **Our objective is to explore a completely new class of molecules as potential targets for bacterial treatment: regulatory ncRNA networks involved in the establishment of bacterial pathogenicity.**

The strength of our consortium rose from the merging of several internationally recognized expertises/competences in complementary research fields including microbial pathogenesis, bacterial physiology, RNA biology, bioinformatics, biochemistry and biophysics. Starting out from a genome-wide search for novel ncRNAs, their involvement in bacterial pathogenicity is being investigated by a variety of tools. A multi-disciplinary approach filters the most promising targets in the regulatory RNA networks involved in bacterial pathogenicity for further exploitation as a **route to novel therapeutic agents against bacterial infections**.

This consortium applied the specific experience of individual partners in the use of complementary and sophisticated approaches. The project significantly contributed **to the conceptual and technological advancement of the state of the art**, and has adapted topical biotechnological, bacterial and genomic research.

1.2 Contractors

Role	No.	Organisation	Short name	Country
CO	P1	Universität Wien	UNIVIE	Austria
CR	P2	Centre National de la Recherche Scientifique	CNRS	France
CR	P3	Insitut Pasteur	IP	France
CR	P4	Uppsala University	UU	Sweden
CR	P5	University of Umeå	UMU	Sweden
CR	P6	The Hebrew University of Jerusalem	HUJI	Israel
CR	P7	punkt international GmbH	punkt	Austria

CO = Coordinator; CR = Contractor

Project Partners












Name	Institution	City	Country
Altuvia Shoshy	The Hebrew University of Jerusalem	Jerusalem	Israel
Charpentier Emmanuelle	University of Vienna	Vienna	Austria
Cossart Pascale	Institut Pasteur	Paris	France
Johansson Jörgen	University of Umeå	Umeå	Sweden
Konrat Robert	University of Vienna	Vienna	Austria
Margalit Hanah	The Hebrew University of Jerusalem	Jerusalem	Israel
Moll Isabella	University of Vienna	Vienna	Austria
Rohner Brigitte - Administrative Manager	punkt international GmbH	Vienna	Austria
Romby Pascale	CNRS - UPR 9002	Strasbourg	France
Schroeder Renée – Coordinator	University of Vienna	Vienna	Austria
Wagner Gerhart	Uppsala University	Uppsala	Sweden
Westhof Eric	CNRS - UPR 9002	Strasbourg	France

1.3 Strategy used towards the achievement of the objectives

The following approaches were used to achieve our goal: to find novel targets for therapeutic intervention in pathogenic bacteria: to search for novel ncRNAs, to indetify their targets, to understand their role in establishing virulence, to evaluate these targets for therapeutic intervention and to suggest potential small molecule ligands to interfere with the target's function.

a) Biochemical, genetic, and computational approaches were used to predict or experimentally identify ncRNAs. A bioinformatics platform was established allowing the prediction for small RNAs in the above-mentioned pathogens.

b) The expression profiles of the ncRNAs were determined under different stress conditions such as heat, oxidative stress, pH, stationary phase, and other conditions that mimic aspects of an infectious situation.

c) Complementary tools were developed to screen for ncRNAs targets, and to evaluate their physiological roles. The identification of target molecules of ncRNAs was accompanied by the delineation of regulatory pathways connecting the ncRNAs to their (virulence) targets, and ultimately the virulent phenotype.

d) The molecular regulatory mechanisms of action of the ncRNA-mRNA and ncRNA-protein interactions was analysed.

e) Structural and mechanistic aspects of the ncRNAs interacting with the targets were studied.

f) The investigation of the biological significance of the ncRNAs and their targets in adaptation to changing environmental conditions, in particular in the host environment, and virulence, constituted the final step in the basic research part of the present project.

g) Small molecule ligands were searched for in computational high-through put screens.

1.4 Performed Work and End Results

1.4.1 Methods developed (WP2)

In order to perform the proposed work, many new techniques had to be developed or adapted. For a better dissemination of the methods present in the consortium a **"tool box"** was established in form of a work package (work package 2). Here we give a few examples of the methods used for this project.

P1/ Renée Schroeder: Genomic SELEX: A method to isolate novel RNAs, with specific affinity to a ligand (small molecule or protein).

Most often, novel RNAs are detected vie RNomics (large scale sequencing of cDNA) or predicted via algorithms, which require as much precise knowledge about the searched RNAs as possible. Through RNomics, the detection of the RNAs is dependent on its expression level. RNAs which are not (highly) expressed cannot be detected. We developed a complementary method, to the mentioned approaches, which is independent on the expression level, and no information is required apart the fact, that they should bind to the bait used for selection.

Genomic SELEX is an experimental procedure for the expression condition independent identification of functional RNAs. RNA libraries derived from genomic DNA are generated via random

priming, PCR amplification and *in vitro* transcription. The library consists of genomic sequences of selected size (50 to 500 nucleotides are flanked by constant sequences required for amplification and transcription. The RNA pool is subjected to several rounds of selection and amplification, whereby various selection criteria are possible. Here we describe as selection criterion the affinity to a protein of interest. With this method, novel functional RNAs can be discovered, nucleic acid-protein interactions can be identified and whole protein-nucleic acids networks can be defined. This method clearly complements *in silico* approaches and experimental procedures, which start from the isolation of expressed RNAs, and is best suited to detect novel functional RNAs, which are differentially expressed and therefore absent from many tissues, or low abundance RNAs.

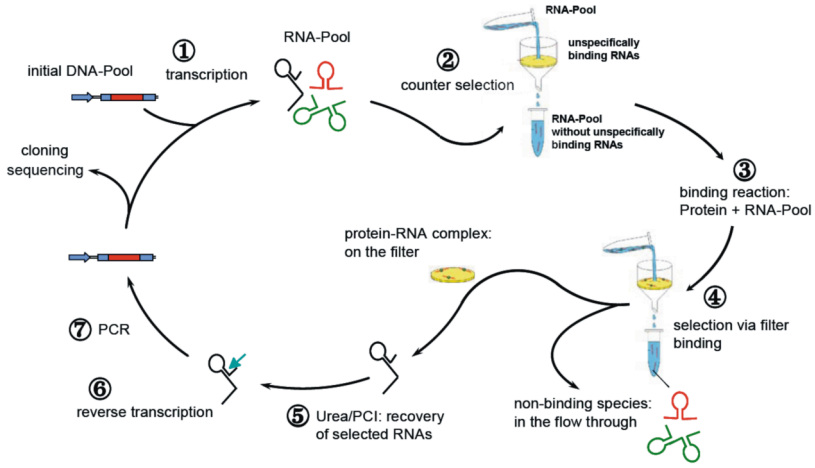


Figure: Overview of the SELEX process. The initial DNA pool, flanked by the fixed primer binding sites and a T7 promoter is transcribed into RNA **(1)**. The resulting RNA library is subjected to a counter selection, in order to remove molecules that unspecifically bind to the membrane **(2)**. After the protein-binding reaction with the counter selected pool **(3)**, sequences binding to the protein of interest are selected via filter binding **(4)**. Thereby protein-bound RNAs are retained on the membrane, whereas non-binding RNAs are eluted. Selected RNAs are recovered via protein denaturation and phenol/chloroform extraction **(5)**, reverse transcribed into DNA **(6)** and amplified via PCR **(7)**. The resulting pool is either subjected to another round of SELEX, or, after sufficient enrichment, cloned and sequenced.

P1/Renée Schroeder: StreptoTag: A method to isolate proteins or RNAs that bind to a specific RNA.

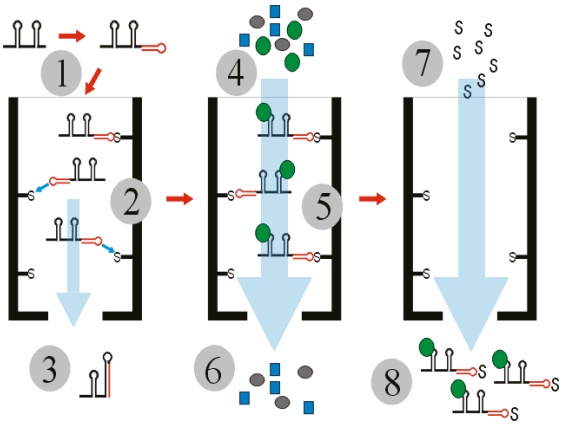


Figure: A fusion RNA between streptoTag and a sRNA from E.coli is created.

2. The fusion RNA binds to a DH-Streptomycin coupled column over the streptoTag
3. Misfolded Fusion RNA does not bind to the column
4. Total E.coli protein is loaded on the column
5. Specific sRNA binding proteins may bind to the fusion RNA
6. Nonbinding proteins are washed away
7. Streptomycin is used for specific elution
8. sRNA + specific sRNA binding protein complex eluted, proteins identified by MassSpec

P1/Isabella Moll: Tools for the identification and analysis of ncRNAs involved in bacterial pathogenicity

(i) To identify potential novel ncRNAs in *Pseudomonas aeruginosa* we made us of the bioinformatic tool RNAz (Washietl et al., 2005). In contrast to the majority of the computational approaches to detect small RNAs in bacterial genomes, the algorithm extracts information based on the evolutionary conservation of RNA structure from a multiple sequence alignment based on the notion that structured RNAs fold into more stable secondary structures than the genomic background sequence of the same composition. RNAz also evaluates the pattern of substitutions in a multiple sequence alignment of related species. Substitutions that are consistent with preserving a base pair (e.g. GCAGU) or that are compensatory (e.g. GCAUA) provide therefore a direct evidence for the conservation of secondary structure. Using NcDNAlign alignments as input 14 candidate loci and using less restrictive MultiZ alignments 136 candidate loci have been predicted. 7 hits were shared between both screens, which have been further analyzed by RT-PCR (published in Sonnleitner et al., 2008).

(ii) Further, we established a novel RNomics approach for the identification of ncRNAs. cDNA libraries from stationary phase cultures of *Pseudomonas aeruginosa* and from cultures exposed to human serum were generated. After size- fractionation and Hfq co-immuno-precipitation 400 clones were sequenced and out of those 11 could be mapped to intergenic regions. The included Hfq co-immuno-precipitation step ensures that RNAs binding to the RNA chaperone Hfq (mainly ncRNAs) will be enriched. Using this approach, we were able to detect 3 novel sRNAs besides the already identified RNAs RsmY and tmRNA, which have been previously described (published in Sonnleitner et al., 2008).

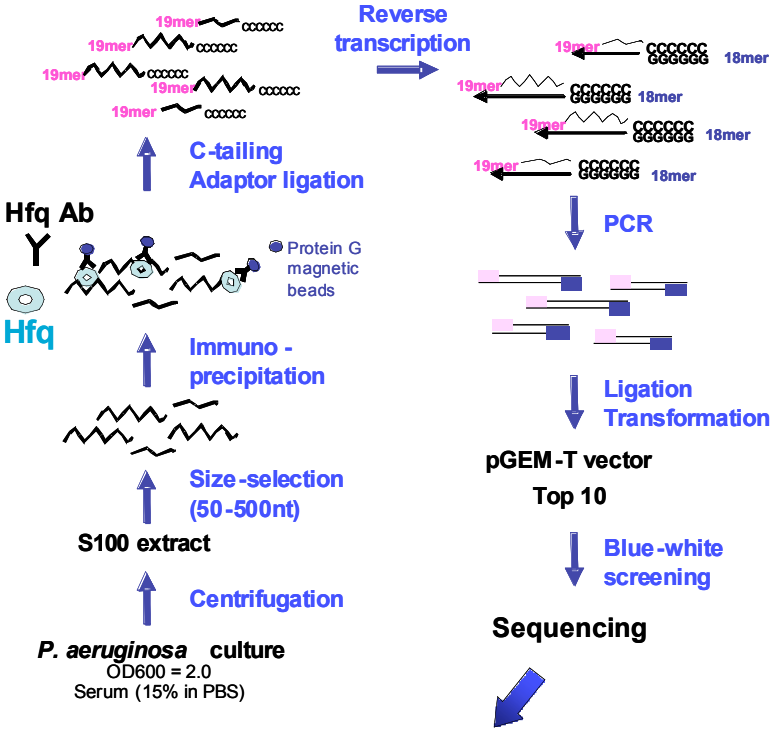


Figure: Schematic representation of the RNomics approach employed to search for Hfq-binding ncRNAs in *P. aeruginosa*

P2/Pascale Romby: A methodology to label surface proteins on living bacteria

P2 contributed to this WP by establishing protocols on methods commonly used in the team (D2.3 Development of tools for ncRNAs characterization) for analyzing the structure of RNA molecules of any size and the interactions between ncRNA and mRNA and/or proteins; for the purification of ribosomes from *E. coli* and *S. aureus* allowing the analysis of ncRNA-mRNA pairing on the formation of the ribosomal initiation complex.

A methodology was also established to label preferentially proteins located at the cell surface of *Escherichia coli* and of *Staphylococcus aureus* derived from the 2-D Fluorescence Difference Gel Electrophoresis (DIGE) methodology. This sensitive approach has been used to monitor the effect of several regulatory RNAs on the synthesis of cell-surface proteins in the two bacteria, *E. coli* (in collaboration with G. Wagner, P4) and *S. aureus* allowing the identification of several novel targets.

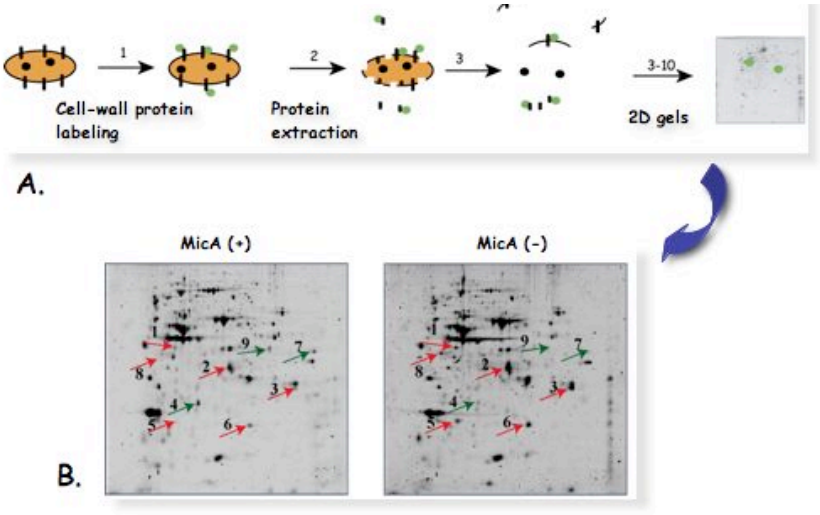


Figure (A) The methodology to label surface proteins on living bacteria, and (B) example of 2D-gel electrophoresis on two different *E. coli* strains which express (+) or not (-) the ncRNA MicA. Proteins for which the synthesis is dependent on MicA, are shown by red (repression by MicA) or green (activation by MicA) arrows.

P2 also contributed to this WP by establishing protocols on methods commonly used in the team (D2.3 Development of tools for ncRNAs characterization) for analyzing the structure of RNA molecules of any size and the interactions between ncRNA and mRNA and/or proteins; for the purification of ribosomes from *E. coli* and *S. aureus* allowing the analysis of ncRNA-mRNA pairing on the formation of the ribosomal initiation complex.

P2/Eric Westhof: The paradise platform

P2 has developed RNA structure modelling and simulation of ribonucleic acids to determine the rules that governs the process of RNA folding and RNA-RNA recognition. A framework called P.A.R.ADIS.E, dedicated to the study of RNA tertiary structures, has been recently built up (http://paradise-ibmc.u-strasbg.fr/bin/view/Main/WebHome). Different graphical tools have been made available to visualize and to build secondary (S2S) and tertiary RNA structures (Assemble). Multiple RNA alignments can be done with RNAlign which aligns RNA molecules against a reference molecule for which the secondary structure has been experimentally established (i.e., by chemical and enzymatic probing). This alignment takes also into account known tertiary in-

teractions, non canonical base pairs, and the base pairing isostericity rules. Thus, RNAlign provides information on conserved sequences and structural motifs. All these tools are linked to RNA algorithms developed in the team (S2S, assemble) or released by other groups (Mfold, Vienna package, CARNAC, MCSym,..).

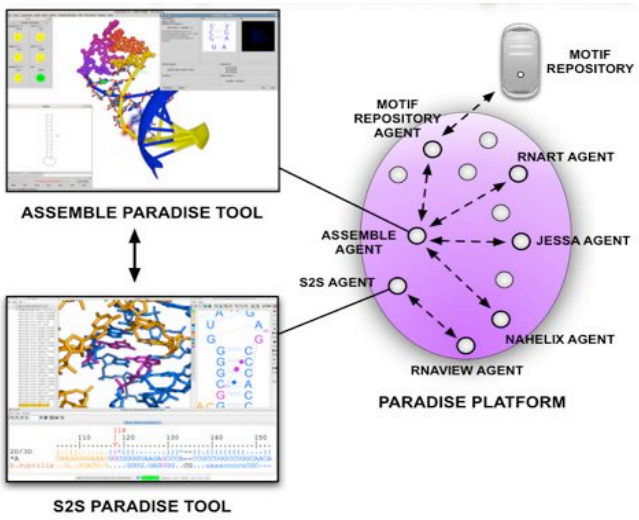


Figure: (A) The paradise platform provides a Java framework to interconnect tools giving information on RNA architecture rules including multiple RNA sequence alignment, RNA secondary structure (S2S paradise tool) and tertiary structure (assemble paradise tool). A web server is available open to BacRNA network and to other labs (http://paradise-ibmc.u-strasbg.fr/bin/view/Main/WebHome).

P2 has also improved protocols for affinity chromatography using biotynilated non coding RNAs to purify targets (proteins, mRNA) in *S. aureus* (with P. Romby) and in *L. monocytogenes* (with P. Cossart, P3).

P3/Pascale Cossart

Identification of ncRNAs

P. Cossart's laboratory has designed a whole-genome tiling microarray "The ListIP Tiling Array" in collaboration with Affymetrix (Santa Clara, CA, USA) covering uniformly both strands of *L. monocytogenes* chromosome. Specifically, the microarray (format 49-7875 with 11 mm features) contains a total of 497,205 probes. Since the chip was thought to be used for different applications, it is divided in three parts. The first part corresponds to the tiling sub-array which contains a total of 345,668 probes covering both strands of the 2,944,528 bp of *L. monocytogenes* EGD-e genome. Each 25-mer probe was 6 tiled each 16 nucleotides across the whole genome, resulting in 9-nt overlaps. The second part correspond to the *L. monocytogenes* gene expression sub-array which contains 62,788 probes grouped into 2,854 probe sets (each probe set includes 11 perfect match and 11 mismatch probes). The last part comprises the gene expression sub-array from the non-pathogenic *Listeria innocua* specie which contains 66,836 probes grouped in 3,038 probe sets. In addition the microarray includes 16,943 antigenomic probes for background calculations and 4,970 control probes. Each sub-array can be analyzed individually using the corresponding sub-arrays maps.

Target prediction:

To predict targets for the novel sRNAs, we used a modified version of the method we previously described (Mandin et al., 2007). We scanned the whole genome of *L. monocytogenes* for RNAs forming stable duplexes with a given sRNA i.e. coding and intergenic regions.

In addition, we developed in collaboration with Eric Westhof biochemical approaches “to fish” RNA or protein targets of sRNA.

P4/Gerhart Wagner contributed to this WP by establishing protocols for the prediction of sRNA targets and for their validation using engineered target gene-reporter gene (translational) fusion plasmids. The model system used was *E. coli*, though both methods can be applied to other bacteria if genome sequences are available and the organism is genetically tractable.

The prediction program developed by Johan Reimegård considers interactions between sRNAs (antisense RNAs) and their targets in regions of low structural stability. Additional score improvement stems from reiterative runs employing genomes of closely related bacteria, since intermolecular base-pairing will be conserved if regulation is the same.

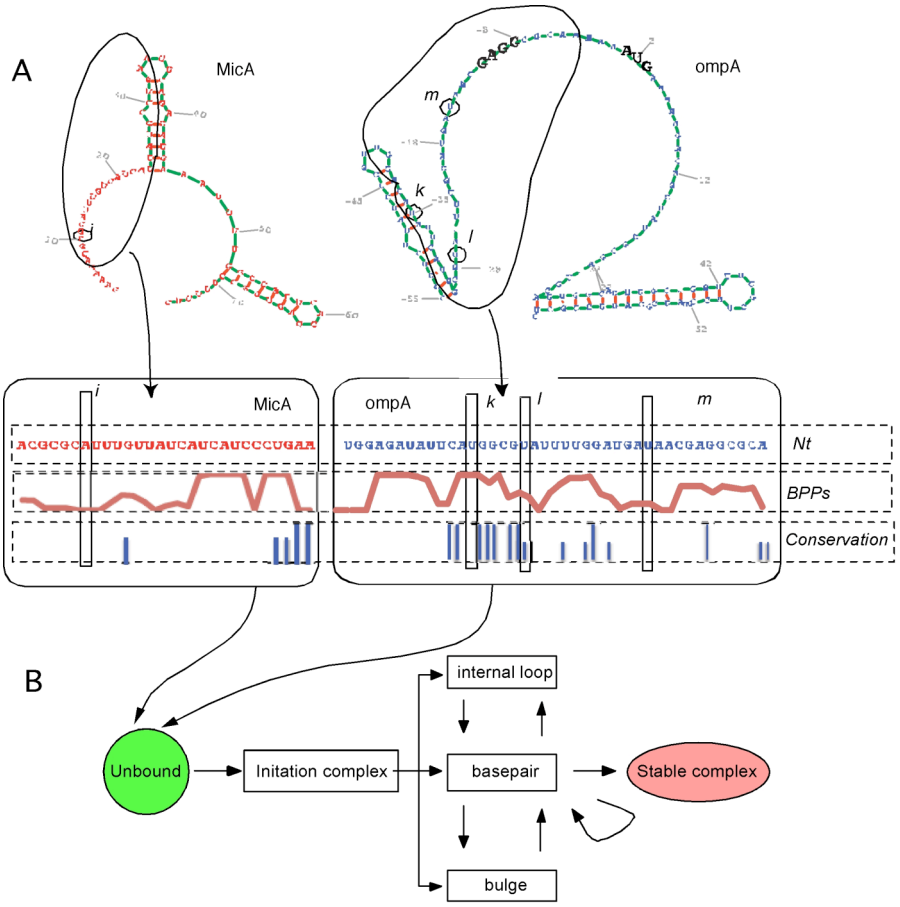


***Figure*: *Schematic outline of the target search algorithm*.** The program uses all extracted mRNA 5'-regions as search space. Details of the procedure are described in the upcoming publication.

For target validation, we created a number of plasmids that carry promoterless *lacZ* or *gfp* coding sequences, however lacking the SD and translational start codon. Suspected target sequences are inserted so that the target gene is fused in-frame with the reporter. The sRNAs in question are encoded from separate compatible plasmids. Measurements of inhibition (or in principle, even activation) of translation is done by colorometric assays or by Western analysis.

In this fashion, we have validated targets for the sRNAs MicA, MicF, OmrA, and OmrB. This approach works for almost all targets, since the interaction site of the sRNA tends to be located within the 5-untranslated region or at least early in the gene. An exception is IstR-1 and its target *tisB.*

Target validation by translational target-reporter fusion constructs works for almost all targets, since the interaction site of the sRNA tends to be located within the 5-untranslated region or at least early in the gene. An exception is IstR-1 and its target tisB. We have recently validated that the csgD gene is targeted by two related sRNAs, OmrA and OmrB. The underlying experiment was conducted such that in vivo base-pairing interaction can be concluded safely; mutations introduced into the target or the sRNA lead to loss of down-regulation of the CsgD-GFP fusion protein, compensatory base changes restored regulation. The read-out was done by Western analysis (antibody against GFP), and non-regulated GroEL was used as a loading control (Holmqvist et al., unpublished)

In collaboration with P2/ P. Romby, we also predicted several MicA-regulated proteins that are outer-membrane located. Validation is partly accomplished, and follow-up work ongoing (see chapter of P2).

P6/Shoshy Altuvia: To learn whether the island-encoded sRNAs of *Salmonella typhimurium* participate in the infection process, we developed of a protocol to detect and measure the expression levels of these sRNAs within macrophages (Padlaon et al 2008). We examined the expression of these genes early and late in infection, by studying their transcripts at one and eight hours post infection. To monitor expression, activated J774 macrophage-like cells were infected with *S. typhimurium* and incubated for the defined periods of time in medium containing gentamicin to eliminate extracellular bacteria. At the end of the incubation periods, the intracellular bacteria were recovered and total RNA was extracted. Because many of the island-encoded sRNAs are expressed at low levels, we monitored the expression of these genes by real-time PCR, an accurate and sensitive technique for measuring low-quantity RNAs.

P6/Hanah Margalit: We developed an algorithm to predict ncRNA encoding genes in pathogenic bacteria, both in the core genome and in the genetic islands of the bacteria. The main steps of the algorithm are: 1) Locate empty intergenic regions. 2) Within these regions predict terminators. These determine the putative 3' end of the ncRNA. 3) Assess the evolutionary conservation of the putative ncRNAs. Final predictions of this module include conserved ncRNAs. In addition, for the genetic islands we carry out predictions in intergenic regions based on the terminator only, without the requirement of evolutionary conservation. For both the core genome and the islands the predictions are further filtered, because some of them might be trailers or leaders of protein-coding genes.

1.4.2 Identification of novel ncRNAs (WP3)

P1/ Renée Schroeder: Cis-antisense RNAs in E. coli, a novel class of large non-coding RNAs.

Using genomic SELEX, we selected RNAs encoded in the *E. coli* genome, that have high affinity to the global regulator protein Hfq. Hfq is known to promote RNA-RNA interactions and many regulatory RNAs are dependent on Hfq to function. With this approach we aimed at identifying novel **regulatory RNAs**. The vast majority of the Hfq-binding RNAs isolated via the genomic SELEX approach lie on the antisense strand of protein-coding genes. We tested the expression of 14 of these novel sequences and surprisingly found that they all give raise to differentially expressed transcripts. This suggests a frequent transcriptional output from the antisense strand of protein coding genes. Preliminary size determination shows that these transcripts are in general over 1 kb long. These *cis*-encoded antisense RNAs **(cis-asRNAs)** may represent a novel class of non-protein coding RNAs in bacteria reminiscent of the natural antisense transcripts (NATs) in eukaryotes.

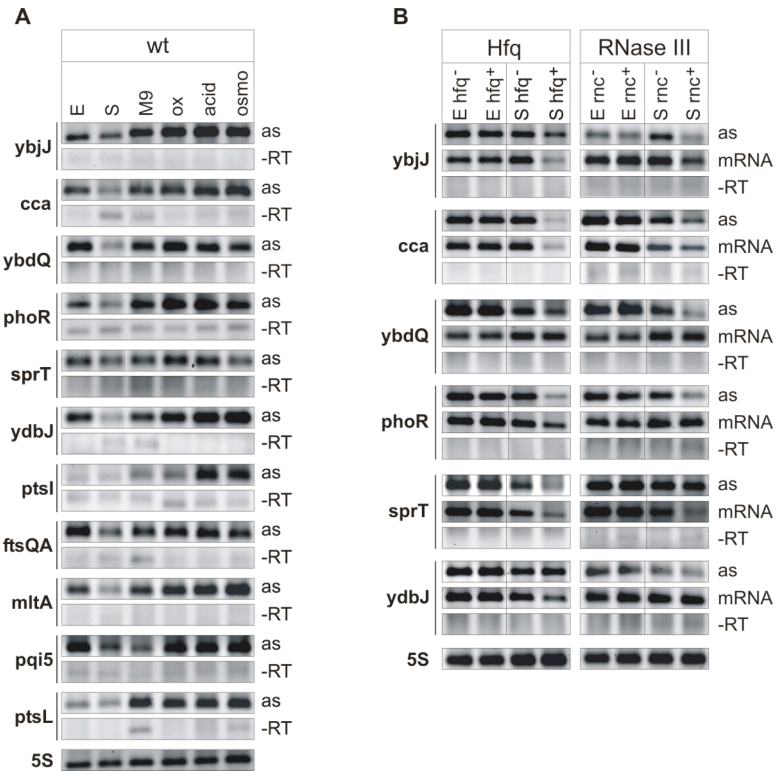


***Figure*: Analysis of cas-RNAs via RT-PCR. 1A:** RT-PCR of 11 casRNAs from total RNA extracts prepared from cells grown at different conditions: E = exponential phase; S = stationary phase; M9 = minimal growth medium; ox, acid, osmo = oxidative, acidic and osmotic stress, respectively. –RT = control of PCR reaction without reverse transcription. **1B:** RT-PCR from total RNA extracts isolated from exponentially growing (E) and stationary (S) cells from E. coli mutants lacking Hfq (left panel) or RNase III (right panel). 5S = RT-PCR of 5S ribosomal RNA as control for RNA loading. Strand-specific RT-PCR was obtained by using only the strand-specific primer during the reverse-transcription reaction: for the mRNA RT-PCR 25 cycles, for cis-asRNAs RT-PCR 35 cycles were performed.

P1/Emmanuelle Charpentier: Identification of novel sRNAs in *S. pyogenes*

To identify sRNAs in *S. pyogenes*, both computational and experimental approaches were employed. The large-scale computational screen consisted of the use of different algorithms, 'QRNA', 'RNAz' and 'sRNAPredict2', to identify intergenic regions (IGRs) likely to encode sRNAs in the genome of the *S. pyogenes* strain SF370 (M1 serotype). In total, 257 individual predictions were analyzed using computational tools (e.g. vector NTI, B-Prom and TransTermHP algoritms) and summarised into 178 loci. 76 (43%) of the 178 loci were further studied using Northern blot analysis under normal growth conditions. From the 76 loci, 28 (37%) sRNAs were expressed, of which five were predicted riboswitches, six were leader elements, four were

functional RNAs, seven were predicted T-boxes and five were novel sRNAs of unknown function. Further characterization of the expressed sRNAs was done by studying their expression patterns in different M serotypes (M1, M3, M5 and M49) throughout the entire growth (Northern blot analysis), determining their intracellular metabolic stability (rifampicin assays), mapping of their 5' and 3' extremities (primer extension; simultaneous mapping of 5' and 3' ends of RNA molecules by circularization – method developed together with the lab of Renee Schroeder (Partner P1) in collaboration with Ciaran Condon, Paris, France) and analyzing their putative secondary structures (M-fold, contra-fold and RNA-fold algorithms). Among the five novel sRNAs, three were determined to be 5'UTR-derived, one was found to be 3'UTR-derived and one, SpyRNA049, was located within an IGR and encoded three sRNAs ending at the same rho-independent terminator. Altogether, our study allowed the identification and characterization of five novel sRNAs and is the first report of expressed riboswitches, T-boxes and functional RNAs in *S. pyogenes*. We also conclude that algorithms need further development to selectively identify trans-acting sRNAs in *S. pyogenes* .

In addition, the improved version of the sRNAPredict2 software (done by the group of Hannah Margalit, Partner P6) was run to predict additional sRNAs in the IGRs of the *S. pyogenes* SF370 (M1 serotype) genome. The results were compared to the computationally predicted sRNAs.

P1/Isabella Moll: Identification of novel ncRNAs in *Pseudomonas aeruginosa* PAO1

First, we identified **three potential ncRNA candidates** in *Pseudomonas aeruginosa* (PaeI, PaeII and PaeIII) employing a classical bioinformatics approach (in collaboration with Birgit Eisenhaber, IMP Vienna), which analyzed intergenic regions for sRNAs up to 300nts flanked by a promoter and rho-independent terminator stem loop.

Second, we employed the novel algorithm RNAz, which is based on the evolutionary conservation of the RNA structure. Using NcDNAlign alignments as input 14 candidate loci and using less restrictive MultiZ alignments 136 candidate loci for ncRNAs have been predicted. **7 hits for novel ncRNAs** were shared between both screens, which have been further analyzed by RT-PCR (published in Sonnleitner et al., 2008).

Third, using the RNomics approach in combination with Hfq co-immunoprecipitation, besides the already identified RNAs RsmY and tmRNA, which have been previously described, we were able to identify **3 novel ncRNAs in** *Pseudomonas aeruginosa* (published in Sonnleitner *et al.*, 2008).

The identified ncRNA candidates have been validated by Northern Blot and/or RT-PCR. We were able to verify the expression of **PaeII**, **PaeIII** and **PhrD** (*Pseudomonas aeruginosa* Hfq-binding RNA) by Northern blot. Expression of PhrX, PhrY, locus 72/101 and locus 102/16 has been detected by RT-PCR. To verify their involvement in pathogenicity the expression the ncRNAs was studied at stationary phase and upon challenge with non-inactivated human serum, conditions that are related to systemic infection. These conditions revealed the induction of PaeIII expression upon encountering infection conditions. In addition, we mapped the 5' ends of PaeIII and PhrD by primer extension analysis.

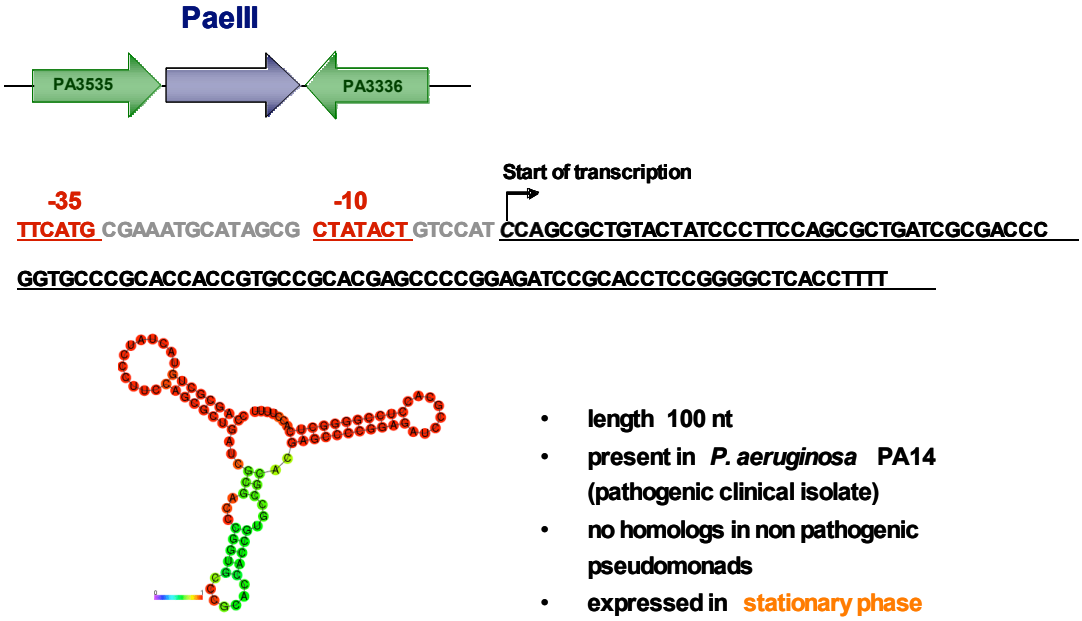


Figure: Sequence, genetic organization and predicted secondary structure of PaeIII ncRNA. The ncRNA is located between genes PA3535 and PA3336 (green arrows). The promoter region of paeIII is indicated in red and the seuence codong for the ncRNA is underlined. The secondary structure predicted by mfold (Mathews et al., 1999; Zuker, 2003) is shown below.

P2/Pascale Romby: Identification of novel ncRNAs in *Staphylococcus aureus*

The group has analyzed intergenic regions (IGR) of *Staphylococcus aureus* N315 strains by using a combination of bioinformatic tools. This analysis integrates the search for orphan terminators and promoters of transcription, conserved sequence and secondary structure motifs, statistical HMM analysis of nucleotide composition, and genomic arrangement. *Staphylococcus aureus*-specific IGR were also taken into account. ApolloRNA (http://carlit.toulouse.inra.fr/ApolloRNA), an extension of the Apollo environment was used to integrate all data and select the candidates for further expression analysis. ApolloRNA interactively visualizes a predicted secondary structure associated to a genomic sub-sequence, and searches for possible RNA/RNA interactions.

All IGR were subsequently analyzed with regard to available data. In the first screening, we did not consider known riboswitches as well as the repeated sequences and STAR elements (as found by HMM and comparative analysis). The expression of 35 IGR was analyzed by northern. These studies were performed in cells grown to exponential and stationary phases in different clinical and mutant strains, as well as under stress conditions including osmotic and oxydative stress, cold and heat shocks, iron limitation, and acidic conditions. These data reveal 11 novel functional IGR and 10 of them encoding for *bona fide* non-coding RNAs. The expression of all RNAs is tightly regulated and four of them are sigma B-dependent. This alternative sigma factor is known to be involved in the regulation of genes encoding for virulence factors.

The team of H. Margalit (Partner 6) has also predicted IGRs of the *S. aureus* NCTC8354 genome. Additional candidates will be analyzed in the nearest future.

P3/Pascale Cossart: New ncRNAs in *L. monocytogenes*

Using this new generation of microarray, P. Cossart's laboratory has identified 29 previously unreported sRNAs in *L. monocytogenes*. Their size varies from 83 to 534 nt:

- Seven are predicted to act as antisense.
- Fifteen of the novel sRNAs are absent in *L. innocua*, pointing to a potential role in virulence.
- Five out of the 29 novel transcripts are predicted to encode small ORFs with appropriated ribosome binding site.
- Several sRNAs were differentially expressed in some of the conditions tested, e.g. 9 were up-regulated in stationary growth phase, 16 were up- and one was downregulated after bacterial growth in blood, 12 were up-regulated in bacteria retrieved from the intestinal lumen.

In *L. monocytogenes*, PrfA is the major activator of virulence gene expression. Other regulators contributing to virulence include the alternative sigma factor B involved in stress response. None of the sRNAs was regulated by PrfA whereas five sRNAs are controlled by SigB.

P4/Gerhart Wagner has attempted to analyze new noncoding RNAs from the major pathogenesis-related plasmid of *Yersinia pseudotuberculosis.* This was conducted in collaboration with the group of Hanah Margalit (P6) who used an sRNA search algorithm to identify putative sRNA genes. Our group conducted Northern analyses on all promising (i.e. not leader or trailer-based RNAs). The outcome indicated that sRNAs were indeed present as predicted. However, most of these, upon inspection, showed structure/ sequence features reminiscent of copy control RNAs similar to CopA from plasmid R1, or were much longer than predicted - indicating them to be parts of mRNAs rather than bona fide sRNAs. See also Table P2. If such searches were continued in the future, deep sequencing approaches might be more promising.

P5/Jörgen Johanson: In a preliminary screen to identify ncRNAs in *Listeria monocytogenes*, we used a comparative homology approach to identify putative ncRNAs in intergenic regions and verified 16 targets by northern blotting. However, 11 of these RNA species were shown to be riboswitches. One suggested ncRNA (rli38) showed an increased expression during growth in H_2O_2 stress condition and in blood. Rli38 is not present in the avirulent *Listeria* species, (*Listeria innocua*). Interestingly, Parts of rli38, can be found in the intergenic regions of other bacterial pathogens, like *Staphylococcus aureus*. A knock-out of *rli38* show a different virulence potential than the *Listeria* wild-type strain.

In addition, we have identified and knocked out several different types of riboswitches, (S-adenosylmethionine, lysine, glycine, adenine binding riboswicthes). We have investigated if some of these riboswitches could have a role *in trans*, affecting expression of targets other than the downstream gene and thereby functioning as "traditional" ncRNAs. From these studies, we have characterized a *trans*-acting SAM riboswitch to have a role in *Listeria monocytogenes* virulence. Absence of the SAM-riboswitch caused *Listeria* to be more infective based on mice LD_{50} experiments and bacterial proliferation in the liver and spleen suggesting a role for the SAM-riboswitch as a virulence repressor.

Absence of the lysine riboswitch causes bacteria to be more motile, an effect that, at least partially, could be suppressed by supplementing the knock-out strain with the lysine riboswitch.

These results suggest a *trans*-regulatory role for the lysine riboswitch in motility and perhaps biofilm production and virulence.

P6/Hanah Margalit: We applied our algorithm to the genomes of the bacteria in the consortium. All predictions (before any filtering) are downloaded on the website of the consortium. Here is a summary table:

Organism	# of predicted ncRNAs
Listeria monocytogenes	303
Listeria monocytogenes 4b F2365	310
Pseudomonas aeruginosa	852
Salmonella typhimurium LT2	831
Staphylococcus aureus NCTC 8325	534
Streptococcus pyogenes M1 GAS	372
Streptococcus pyogenes MGAS5005	335
Yersinia pseudotuberculosis IP32953 plasmid	21
Yersinia pseudotuberculosis IP32953	973

P6/Shoshy Altuvia: Using a novel computational-experimental screening strategy we identified 19 unique sRNA genes encoded within the genetic islands of *S. typhimurium*. The characterization of the newly discovered island-encoded sRNAs demonstrated that many of these genes are expressed under unique stress conditions. Some of the conditions such as high osmolarity, extreme pH, starvation, oxygen limitation, oxidative stress and conditions of iron and magnesium limitation are reminiscent of the environments *S. typhimurium* encounters upon invasion of intestinal epithelium, or within macrophages. Several of these sRNA genes are indeed induced when Salmonella resides within macrophages. Characterization of one sRNA IsrJ, shows that is affects invasion as well as translocation of effector proteins into epithelial cells. Consistent with the above phenotype, the analysis of the array data of IsrJ indicated that it up -regulates invasion related factors and fimbriae encoding genes and down regulates genes of chemotaxis and the flagellar apparatus. We are now in the process of verification of that data.

1.4.3 Identification of ncRNA targets (WP4)

P1/Emmanuelle Charpentier: Functional analysis of selected novel sRNAs in *S. pyogenes*

Several sRNAs were selected for further functional analysis: (i) their possible functions in the pathogenicity of *S. pyogenes* including virulence, adaptation to stress and host, and physiological essentiality, (ii) the identification and validation of their putative targets (mRNAs and proteins).

From the computational screen described above, expression of selected sRNAs from the 28 validated sRNAs was analyzed under stress conditions (heat shock, salt and acid stress) (WP3), however no significant changes of expression were revealed by Northern blot analysis.

SpyRNA014: SpyRNA014 was predicted by three different computational algorithms and the experimental RNomics screen (WP2, WP3). Several predictions next to *scpA*, a gene encoding the virulence factor C5a peptidase, resulted in the mapping of an sRNA, SpyRNA014, originating from the 5'UTR of the *scpA* mRNA (WP2, WP3). Northern blot analysis revealed a similar growth phase dependent expression pattern when comparing the *scpA* transcript with SpyRNA014 (WP3, WP4). SpyRNA014 was already mentioned in the literature as a truncated *scpA* mRNA, possibly generated through a transcription termination mechanism (WP3, WP4). SpyRNA014 was still included in this study since the mechanism of *scpA* regulation is thus far not elucidated and other functions of SpyRNA014 cannot be ruled out.

SpyRNA049: SpyRNA049 was predicted by three different computational algorithms and the experimental RNomics screen (WP2, WP3). SpyRNA 049 is encoded from a small IGR of the *S. pyogenes* SF370 genome. SpyRNA049 is transcribed as three transcripts, independently of the two adjacent ORFs (Northern blot, mapping by primer extension and self-circularization) (WP2, WP3). Because of the lack of putative translational signals in the sequence of SpyRNA049, this sRNA can be considered as a typical non-protein-coding RNA. Further a stability of more than 30 min was shown for two of the three transcripts (rifampicin assays) (WP5). Using a computational approach (software developed by the group of Pascale Cossart, P3) to search for putative mRNA targets of SpyRNA049 led to *cfa* mRNA, encoding the CAMP factor involved in streptococcal pathogenicity. Candidate SpyRNA049 was predicted to bind to an upstream region of the *cfa* ORF (5´ mRNA) (WP4). Analysis of the interaction of SpyRNA049 with the *cfa* mRNA by gel shift assays is in progress to validate the target (WP4, WP5). To assess a possible biological function of SpyRNA049, a SpyRNA049-null strain was constructed in strain SF370 and the mutant strain was compared to the wild-type parent in internalization in epithelial cells and survival in macrophages (WP2, WP3, WP4). However, no significant differences could be observed.

P1/Isabella Moll: Identification of targets controlled by ncRNAs involved in virulence in *Pseudomonas aeruginosa*

To identify possible targets for the validated ncRNAs we performed proteomic studies. 2D gel-electrophoresis upon over-expression of the potential ncRNA **PaeIII**, which was shown to be induced under infection conditions, revealed activation of the possible target gene PA1202, encoding a putative hydrolase, while the expression of ORF4739 and the gene encoding protein PurE was repressed. In addition, a transcriptome analysis upon *paeIII* over-expression has been performed. This study revealed that expression of components of the MexXY multidrug efflux system as well as that of the Type III secretion apparatus (important virulence genes) is increased, whereas expression of genes involved in pyocin and amino acid biosynthesis as well as genes involved in anaerobic respiration is decreased. Direct targets are under investigation. At the moment transcriptome analysis comparing the wild type *Pseudomonas aeruginosa* strain PAO1 to knockout strains for the ncRNAs PaeII and PhrD and inducible over-expression strains are under investigation to search for their targets.

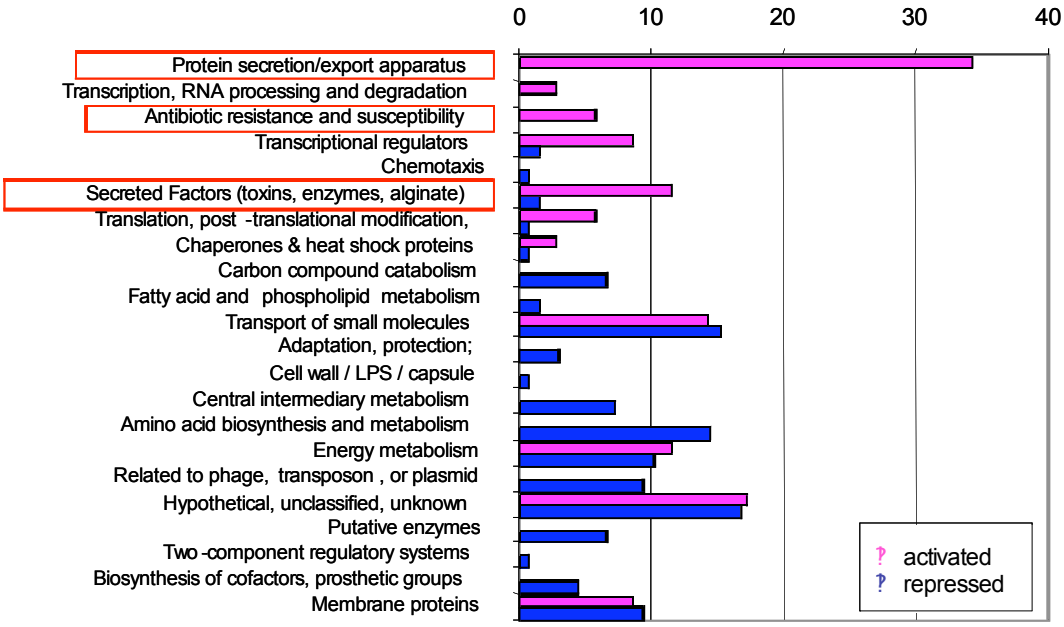


Figure: Identification of PaeIII targets by transcriptional profiling upon overexpression of *paeIII*. The pink and blue bars represent the genes activated or repressed upon induction of *paeIII* expression in a *P. aeruginosa paeIII* knock out strain.

In addition, we tested for RsmY targets, which are independent of RsmA regulation. Transcriptome analysis of a *Pseudomonas aeruginosa rsmA/rsmY* double mutant upon *rsmY* overexpression has been performed. These studies revealed that over-expression of the *rsmY* gene yielded a higher expression of genes involved in carbon compound catabolism (*ant* and *cat* clusters), whereas genes related to energy metabolism were shown to be down regulated. Direct targets are under investigation.

New targets involved in virulence of *S. aureus*

P2/Pascale Romby has experimentally validated new targets of the quorum sensing dependent RNAIII and of several ncRNAs (see WP3) from *S. aureus*. (A) Targets of RNAIII: several mRNA targets (encoding for virulence factors) have been initially predicted by an *in silico* analysis which takes into account binding energy properties, and structures of the mRNAs. Experimental validation was done for two mRNAs encoding for coagulase, a main virulence factor, and for the peptidoglycane hydrolase LytM. This study includes expression analysis of the endogenous mRNAs and of a reporter translational gene fusion ((the regulatory region of *coa* mRNA was fused in frame with the coding sequence of *lacZ*) in wild type and mutant strains (deleted of RNAIII). The mutant strain was also complemented with wild type or mutant RNAIII expressed from a plasmid. Proteomic analysis and phenotypic assays on different strains (WT, ΔRNAIII, ΔRNAIII-ΔlytM) show that RNAIII-dependent repression of *lytM* mRNA is required to maintain the integrity of the cell wall of the bacteria. Comparative labelling of the surface proteins in the WT and ΔRNAIII strains reveals that RNAIII modifies the surface composition, and regulates other membrane proteins (ABC transporters, lipoyl synthase). It remains to be known whether these effects are direct or indirect. (B) Targets of ncRNAs: a combination of strategies has been used to identify the functions regulated by ncRNAs. Deletion of four of the ncRNA genes (RsaA, RsaE, RsaG and RsaH) has been done. Phenotypes of these mutant strains have been analyzed by monitoring their effect on growth rate in BHI medium. Proteomics and transcriptomic

analysis have been assessed for the mutant strains RsaE and RsaH. These data show that RsaE regulates the expression of several proteins involved in the TCA cycle and glyoxylate metabolism, and nutriment availability while RsaH regulates the expression of several virulence factors. *In silico* search was performed to predict base pairing complementarities with the ribosome binding site of mRNAs for which the expression is dependent on RsaE or RsaH.

P2/Pascale Romby's team has previously shown that the double-stranded specific RNase III and the regulatory RNAIII co-ordinately repress the synthesis of several virulence factors. Comparative proteomic analysis was recently performed on the WT and the isogenic Δrnc revealing that the absence of RNase III alters the synthesis of several cytosolic proteins. A flag-tag RNase III carrying a point mutation in the catalytic site was recently expressed in Δrnc strain. This mutated protein can still bind to RNA but has lost its enzymatic activity. The protein carries a flag-tag in order to immunoprecipitate the protein from *S. aureus* and to identify the bound RNAs by high throughput cDNA pyrosequencing. These experiments should give a rough overview of the roles that RNase III may have in the control of *S. aureus* gene expression, and may reveal novel mRNA-ncRNA interactions as potential substrates.

P3/Pascale Cossart: New targets involved in virulence of *Listeria monocytogenes*

Using *in silico* analysis, several sRNAs were found to interact with other sRNAs. Most of the sRNA "mRNA targets" identified corresponds to genes involved in bacterial metabolism. In contrast, several sRNAs, *rli28*, *rli32*, *rli34*, *rli38*, *rli49* and *rli50* paired with genes potentially more directly involved in virulence like surface proteins of the internalin family.

P4/Gerhart Wagner: New targets involved in virulence in E. coli

and his group has experimentally validated several new targets for sRNAs in *E. coli.* The reasoning in selecting certain sRNAs for analysis was as follows. In pathogens like *S. aureus* and *Listeria monocytogenes*, some sRNAs are known to be direct regulators of virulence. In pathogenic and non-pathogenic *E. coli* strains, current knowledge suggests that a multitude of stress responses are directly or indirectly subject to sRNA regulation. These conditions are pertinent to a pathogeic lifestyle. Thus, we have analyzed sRNAs whose expression patterns suggested stress response association. Thus, IstR is associated with the SOS response, MicF and MicA are part of the membrane stress response, and OmrA/B is involved in control of biofilm formation - usually associated with virulence traits. We have published the first MicA target, encoding the mRNA for the outer membrane protein A (OmpA). IstR-1 targets *tisB*, encoding an SOS-induced small toxin, MicF targets *lrp*. This latter gene encodes a transcriptional regulator, which suggest an interesting feed forward loop in action.

OmrA/B, interestingly, targets the master regulator of curli (a surface bound appendix structure involved in biofilm formation). This could ne initially demonstrated by overexpressing either of the two RNAs from plasmids in a ΔomrA/B strain background. Plating such strains, and control strains lacking overexpressing plasmids, on Kongo red plates showed clearly that high OmrA/B levels turn colonies white - indicating a lack of curli. The same experiment conducted by plating on calcofluor plates showed effects on cellulose production (light blue/ dark blue color). Since both curli and cellulose were downregulatd, this pointed towards a central regulator being affected. This in turn suggested CsgD as the target, in line with bioinformatics target predictions (see above).

P5/Jörgen Johanson: Our group together with Institut Pasteur (P3) has identified a direct mRNA target (*prfA*) of a *trans*-acting SAM riboswitch by bioinformatic, genetic and biochemical methods. PrfA is the master regulator of virulence in *Listeria monocytogenes.* The *trans*-acting SAM riboswitch appears to be binding to the distal part of the 5´-untranslated region in front of the *prfA* mRNA. Several indirect targets of the SAM-riboswitch were identified by a preliminary gene-array study, among them genes regulated by PrfA. PrfA is the master regulator of virulence in *Listeria monocytogenes* controlling almost all virulence traits and a strain lacking *prfA* is completely attenuated in virulence as has been shown by Cossarts laboratory (P3).

Using, bioinformatics, gene-array and 2D-gel techniques, several (in)direct targets of the ncRNA, Rli38 were identified. These targets have been analyzed but none of them appears to be direct targets of Rli38.

We have studied a possible regulation of motility genes by a *trans*-acting Lysine riboswitch by using affymetrix gene-chips. By this approach, we identified 51 mRNAs displaying an altered expression compared with the wild-type strain. Many of these genes were involved in motility, but despite screening for several likely candidates, no direct target for the lysine riboswitch has been found.

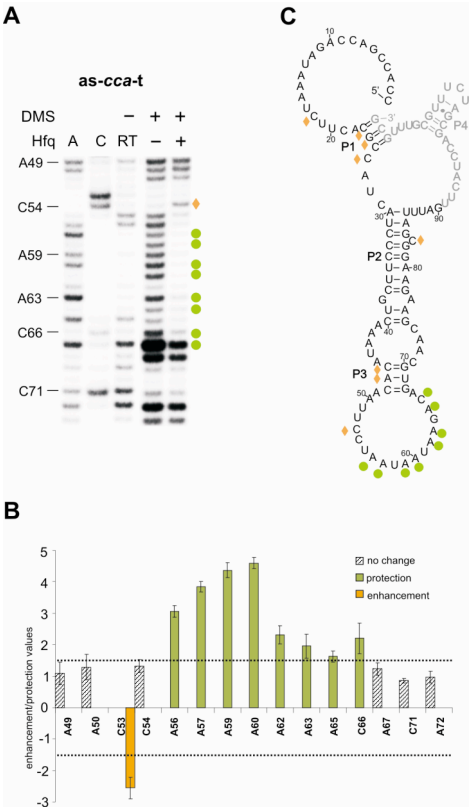
P6/Hanah Margalit: New targets involved in virulence

Prediction of targets of bacterial ncRNAs is a very difficult, as the ncRNA is between a few dozens to a few hundreds nucleotides long, and the base-pairing with the mRNA target may occur anywhere along the ncRNA. We developed and tested along the project three modules for target prediction. The first involved prediction of RNase III cleavage sites. It was based on a training set of experimentally verified cleavage sites and a probabilistic model incorporating the features of these sites. The second module took into account the free energy gain of the mRNA upon base-pairing with the ncRNA. Thus, it considered both the free energy of the folded mRNA itself and the free energy of the complex. The third module, which looks very promising, looked for evolutionarily conserved sub-sequences in the ncRNA, and searched for their matches in mRNA sequences. For a number of known targets, this match coincides with the Shine-Dalgarno sequence. We developed ways to assign a statistical significance to the ncRNA-mRNA matches, and can select targets with binding in the Shine-Dalgarno region, only when they are statistically significant.

P6/Shoshy Altuvia: Although the computational screen focused on orphan terminators located in intergenic regions, the experimental mapping of the sRNAs showed that 11 out of the 19 that were confirmed share sequences with at least one of the flanking genes. We examined the expression of genes that overlap the newly identified sRNAs and identified 7 target genes to be either up or down regulated by their cis encoded sRNA regulators (Padalon et al 2008).

1.4.4 RNA structure / interaction with ligands- Regulatory function/mechanisms- Regulatory networks (WP5)

P1/ R. Schroeder: Identification of the high affinity Hfq-binding motif



From our genomic SELEX experiment, we first sequenced 170 RNAs, which were obtained by individual cloning of the SELEX pool. Through deep sequencing using 454, we obtained further 10.000 Hfq binding RNAs. Computational analysis of all these sequences clearly revealed one dominating motif, 5' ANAANAA 3' to confer high affinity to Hfq. We validated this motif to be a *bona fide* Hfq binding site by performing structural probing and chemical footprinting with a series of obtained RNA clones.

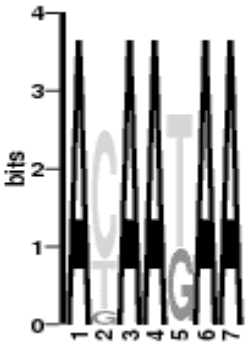


***Figure*: The *in vitro* DMS analysis** reveals changes in RNA accessibility upon Hfq binding to as-*cca*-t. (A) A representative region of an 8 % denaturing gel shows the cluster of protections (green dots) and one enhancement (orange diamonds) upon Hfq addition (+Hfq lane; [0.4 μM Hfq_6]). A, C are sequencing lanes, RT is the control not treated with DMS, - or +Hfq lanes are treated with DMS. (B) Calculated protection and enhancement (ratio of DMS –Hfq/DMS +Hfq), defined by a value of ≥ 1.5 for protections (green) and ≤ -1.5 for enhancements (orange) are presented for the selected region. Dashed bars correspond to nucleotides without changes in the DMS pattern. (C) Predicted secondary structure of the RNA obtained from the cis-asCCA RNA. Green dots represent positions protected from DMS modification by Hfq, while orange dots are enhancements. These experiments confirm the above mentioned motif to be the Hfq binding site.

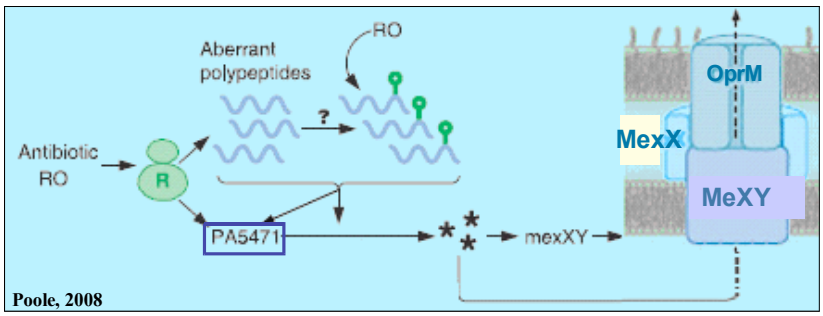
P1/Renée Schroeder: Isolation of sRNA-binding proteins from *E. coli*

Many bacterial sRNAs regulate gene expression enabling cells to adapt to various growth conditions. Assuming that most RNAs require proteins to exert their activities, we purified and identified sRNA-binding factors via affinity chromatography and mass spectrometry. We consistently obtained RNA polymerase ß-subunit, host factor Hfq and ribosomal protein S1 as sRNA-binding proteins in addition to several other factors. *In vivo* binding of some of the sRNAs to the RNA polymerase was confirmed via co-immuniprecipitation in cell extracts prepared from different growth conditions. Most importantly, we observed that RNA polymerase not only binds several sRNAs but also reacts with them, both cleaving and extending the RNAs at their 3' ends. The fact that RNA polymerase reacts with sRNAs maps their interaction site to the catalytic core of the enzyme. We further performed genomic SELEX to isolate RNA polymerase-binding RNAs and obtained a large number of *E. coli* sequences binding with high affinity to this enzyme. Our observations show that the RNA polymerase is able to interact with many different RNAs and we thus speculate that riboregulation of transcription might be a more general phenomenon than originally anticipated.

P1/Isabella Moll: Regulatory mechanisms of ncRNAs involved in bacterial pathogenicity and RNA structure

Possible regulatory mechanisms and the involvement of the ncRNA in a general regulatory network are studied for **PaeIII**. Several results of our studies indicate that this ncRNA might be destabilized by Hfq and regulated by RpoS. As we have shown that overexpression of the *paeIII* gene affects virulence genes, the direct interaction of PaeIII with virulence genes (*mexXY*, genes encoding the TIIISS, *hcn*) and virulence gene expression regulators (MexZ, LadS, SigX, MarR) is under investigation.

PA number	Gene Title	Gene Symbol	Fold Change
PA2018	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter	mexY	5,3
PA2019	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein p	mexX	7,3
PA2020	probable transcriptional regulator mexZ	mexZ	2,2
PA5470	probable peptide chain release factor	--	6,8
PA5471	hypothetical protein	--	4,6



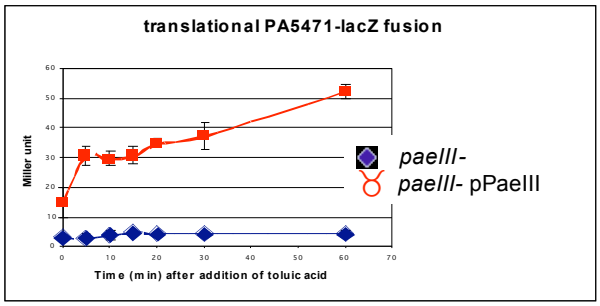


Figure: Validation of the potential PaeIII target PA5471, which was identified by transcriptional profiling to be upregulated upon overexpression of *paeIII*. The protein encoded by gene PA5471 is involved in the formation of a multi-drug efflux pump as indicated in the schematic representation (taken from Poole, 2008). Measuring the β-Galactosidase activity of the translational PA5471-LacZ fusion in a *P. aeruginosa paeIII* knock out strain with (red line) or without (blue line) *paeIII* overexpression revealed the induction of PA5471 expression by the ncRNA PaeIII.

P2/Pascale Romby: **NcRNA Interactions in S. aureus.** The interactions between the quorum-sensing dependent RNAIII and two mRNA targets (*coa*, *lytM*) have been probed by chemical and enzymatic probing. For *coa* mRNA, structure probing shows that two distant domains of RNAIII bind to the mRNA to cover the ribosome binding site as well as part of the coding sequence. This stable complex is rapidly formed (as measured by band shift analysis) and is sufficient to prevent the formation of the initiation ribosomal complex (as monitored by toeprinting assays). As for many other targets of RNAIII, the double-stranded specific endoribonuclease III (RNase III) cleaves the formed complex and is required for efficient regulation. (B) We have analyzed in details the recognition of *S. aureus* RNase III with several mRNA-ncRNA com-

plexes, and show that the enzyme recognizes unusual topologies such as kissing loop complexes. The topology of two different RNAIII-mRNA complexes recognized by RNase III was also analyzed by three-dimensional modeling in the team of E. Westhof. This study shows that coaxial stacking of two helices induced by the loop-loop interaction forms a long helical structure that is efficiently recognized and cleaved by the homodimeric enzyme. Thus, the enzyme can be useful for probing *in vitro* RNA-mRNA complexes. (C) Our data combined with other published works were used to reconstitute the RNAIII-dependent regulatory networks involved in virulence of *S. aureus*.

The secondary structure of several newly-identified Rsa ncRNAs was determined as well as sequence alignment (made by PARADISE, P2/E. Westhof). In most of the ncRNAs, a conserved C-rich sequence is always found in unpaired regions (repeated 1 to 4 times), suggesting that these regions are functionally relevant. *In silico* prediction suggests that many of these ncRNAs target mRNAs through base pairing. Validation of several of the mRNA-ncRNA interactions is under study. Transcriptomic analysis shows that RsaE represses the *opp3* operon. *In vitro*, RsaE binds rapidly and forms stable complex with *oppB* mRNA, and the formed complex prevents the formation of the initiation ribosomal complex. This strongly suggests that RsaE regulates the expression of opp-3 operon which was shown to be the main Opp system supplying the cell with peptides as nutritional sources.

P2/Eric Westhof: Molecular modelling was carried out on a large regulatory non coding RNA, composed of a twin-ribozyme. Comparative analysis of the structures provide the basis for an evolutionary mechanism that can be applied to any other large non coding RNAs such as the ribonuclease P or the so-called riboswitches.

In collaboration with S. Altuvia (P6), a structure model was derived for *E. coli* SraF based of sequence and structure alignment and chemical probing.

P3/Pascale Cossart: NcRNA interations in *Listeria monocytogenes*

Besides sRNAs acting in *trans*, important regulatory mechanisms are mediated by *cis*regulatory RNAs: These can either be long 5'UTRs (untranslated regions) which can form alternative conformations and modulate expression of the associated downstream genes or cis-antisense RNAs controlling expression of the mRNA transcribed from the opposite strand. We have uncovered far more diverse types of RNAs than expected: besides 50 non-coding RNAs and 53 cis-regulatory RNAs, we identify long overlapping 5'- and 3'-UTRs, and long antisense RNAs covering several ORFs.

In particular, we emphasize a novel regulation mechanism controlling the listerial flagellum expression through the SigB-dependent transcription of the MogR repressor and the post-transcriptional mRNA degradation of the flagellum genes due to the long MogR 5'-UTR antisense.

P4/Gerhart Wagner: **NcRNA interations in E. coli**

Particular emphasis was placed on mechanistic studies of two sRNA-target systems, IstR-1-*tisB* and omrA/B-*csgD*. Both turned out to be very interesting and suggested new mechanisms that before have not been encountered. In the first case (see Dissemination Report) it was shown that the toxin-encoding *tisB* mRNA was present as three different species, only one of which was translationally active. In short, a processing event was required to enable toxin translation.

The active mRNA is inhibited by IstR-1 binding to a region far upstream oft the *tisB* RBS. We proposed a model in which so-called standby ribosome binding is required to promote translational initiion at the structurally sequestered - and thus normally inactive - *tisB* start site. IstR-1 prevents standby and thereby inhibits translation. Also OmrA/B target regions within its target mRNA far upstream of the *csgD* RBS. However, so far, standby binding does not seem to be involved. Instead, the upstream target region may be a translational enhancer element which communicates with the RBS to facilitate transcription. Therefore, interaction with the sRNAs appears to break this communication and downregulate translation. This was suggested by swappig experiments in which a heterologous RBS was introduced into the target-reporter fusion plasmid. This new reporter construct was no more controlled by the two sRNAs in spite of an unaltered upstream target region.

Additional work was concerned with the structures of the interacting RNAs. In the IstR-1/ *tisB* case, enzymatic and structural mapping, combined with phylogenetic analyses (giving consensus mRNA folding schemes by taken constraints into account) was instrumental in formulating the regulation model shown below.

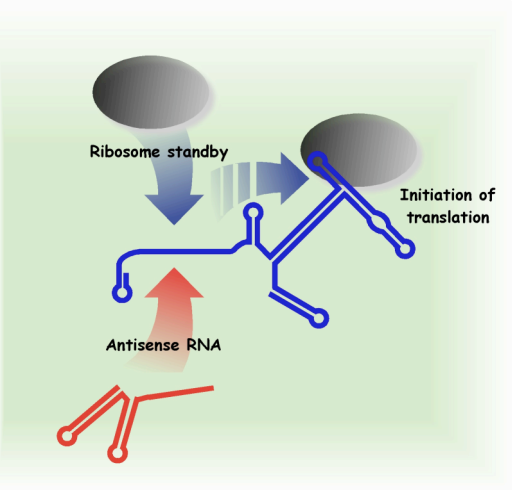


Figure : *Model for Istr-1-dependent regulation of TisB toxicity*

The Figure shows an oversimplified model of how the active *tisB* mRNA can either be translated - based on ribosome standby in a low-structure domain, or be inhibited by the antisense RNA sequestering the same sequence. If standby occurs, ribosomes can await transient opening of the strong secondary structure that otherwise prevents TisB translation.

A final project with a strong mechanistic slant addresses the role of the so-called CRISPR elements that are present in bacteria and archea. Here, several CRISPR-associated *cas* genes encode proteins that process a CRISPR transcript. The unique units, called spacers, (bordered by identical repeats) can target incoming phages or plasmids for inhibition. Even though the general role of this system as an acquired immunity determinant is well-supported, its mechanism of action is not known. Within this project, we have created CRISPR-target-encoding plasmids, based on a GFP readout. We are in the process to experimentally distinguish whether the molecular target of CRISPR-*cas* is RNA or DNA.

P5/Jörgen Johansson: Riboswitch interactions in Listeria monocytogenes: A gene encoding an arsenate reductase (*lmo2230*) which is up-regulated in a strain lacking the *trans*-acting SAM-riboswitch, has previously been show to be activated by PrfA. We therefore tested if the *trans*-effect of the SAM-riboswitch could be through the level/activity of PrfA. By performing computational, biochemical, gene-fusion and genetic experiments, we have determined that the *trans*-acting SAM-riboswitch binds to the 5´-untranslated RNA lying in front of the *prfA* mRNA. By mutational experiments, it appears that the binding site is quite distal from the ribosomal binding site, suggesting a novel type of mechanism. The interaction between the *prfA*-RNA and the *trans*-acting SAM riboswitch blocks PrfA production but does not seem to destabilize the

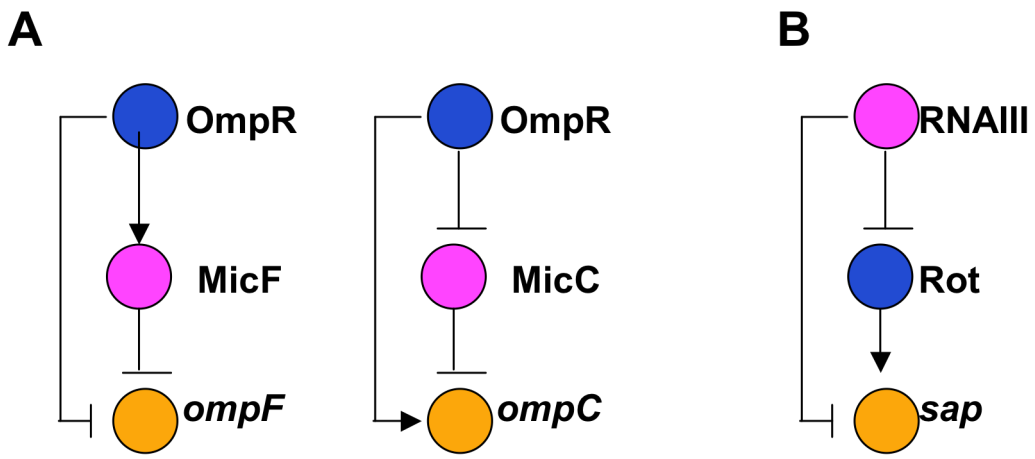
prfA-mRNA in the same range (WP 5.3). Intriguingly, expression of the *trans*-acting SAM riboswitch is positively controlled by PrfA. This leads to a negative feedback loop, where an overexpression of PrfA results in higher amounts of the *trans*-acting SAM riboswitch which in turn down-regulate PrfA expression (WP 5.4).

In silico structure predictions of Rli38 using various computer programs reveal several hairpins. *In vivo* stability of rli38 and different SAM riboswitches suggest a large heterogeneity in stability, with half-life ranging from 25 seconds to several minutes (WP 5.2).

P6/Shoshy Altuvia:

We have been working on the mechanism by an RNA regulator controls the expression of its downstream gene. We found that the E. coli RNA regulator SraF regulates the translation of the downstream gene alx in response to extreme alkaline conditions. Single point mutations in the sraF-alx readthrough transcript, together with chemical and enzymatic probing, demonstrate that it can form two distinct alternative structures, one of which is translationally active. The formation of the active structure occurs while transcription progresses under high pH conditions and involves pausing of RNA polymerase at two distinct sites.

P6/Hanah Margalit: We applied computational analyses to study the dynamics of regulation by ncRNAs compared to other regulation types, such as transcription regulation and protein-protein interaction. To this end, we described the various regulatory mechanisms by mathematical models, followed by simulations using average kinetic parameters based on experimental data in prokaryotes. We showed that there are measurable qualitative differences between the three regulation mechanisms, both in response time and in effectiveness. Regulation by ncRNAs was shown to be advantageous when fast responses were needed, for example, in response to stress. We also integrated the network of regulation by ncRNAs in Escherichia coli with the network of transcription regulation, and found complex regulatory motifs that involve both transcriptional regulation and post-transcriptional regulation by ncRNAs. These included mixed feedback loops and mixed feed-forward loops, which were shown by us to be of advantage in particular cellular situations. We identified such mixed regulatory circuits in the pathogenic bacteria, as illustrated in the Figure below.



S. typhimurium *S. aureus*

Figure: Mixed regulatory circuits involving transcriptional regulators and ncRNAs.

1.4.5 Target validation and ligand-binding analyses (WP6)

P1/Renée Schroeder: The meta-structure approach was adapted to RNA systems and applied to a selection of RNAs provided by the Schroeder group. It was found that (i) the RNA meta-structure provides an efficient additional computational tool for the analysis of RNA sequences (complementing conventional bioinformatics approaches) and that (ii) the approach offers direct access to RNA functional sites, as demonstrated with applications to riboswitch systems and other RNA-ligand systems.

P1/Emmanuelle Charpentier: The FMN sRNA is located downstream of ORF SPy0373, a predicted riboflavin transporter and might affect its expression via a termination-antitermination mechanism. *S. pyogenes* can not synthesize riboflavin and must rely on an efficient transport system that allows uptake of exogenous riboflavin from external sources, such indicating an essential function of SPy0373 in *S. pyogenes*. Expression of SPy0373 at the transcriptional level is induced when cells are switched from a medium containing riboflavin to a medium depleted in riboflavin, thus confirming the likely essentiality of the predicted FMN sRNA target, Spy0373.

P1/Robert Konrat: The meta-structure approach was applied to the putative tryptophan transporter Spy_1016 (in cooperation with E. Charpentier (P1)). Some of the identified ligands were commercially available and have been purchased for testing (in the lab of E. Charpentier (P1)). Only active ligands will be further investigated by NMR binding studies using recombinant proteins. Additionally, the meta-structure approach was used to calculate/analyze the entire proteome of *S. pyogenes*. This information will be subsequently used to screen for homologues for which no significant sequence similarity is found.

P3/Pascale Cossart: We have applied the meta-structure technique to analyze the protein-protein interaction motif between the protein Internalin from *Listeria Monocytogenes* and the Mucin protein MUC2 (P. Cossart). The new meta-structure approach provided structural information about Internalin, which was in agreement with the recently crystal structure. Additionally, the meta-structrue analysis revealed valuable (and unprecedented) information about the Internalin binding partner MUC2. We could show that MUC2 comprises several van Willebrand factor (vWF) domains, a subsequent (sequence-based) protein interaction analysis revealed the vWF domains as the relevant protein interaction site(s). This novel information can now be used to identify potential inhibitory peptides and/or small molecule ligands to block this important protein interaction event. In order to verify these findings a joint effort with P.Cossart's group was started to express Internalin in an *E.coli* expression system. First attempts were not successfull and have to be repeated using different Internalin constructs. This is planned in cooperation with P.Cossart's group. Additionally, 22 proteins from *L. monocytogenes* for which conventional bioinformatics analysis did not reveal any meaningful data were investigated by our approach. Interesting similarities were found for several proteins and this information can be used for the planning of future biological experiments.

P5/Jörgen Johansson: We have identified *prfA*, the direct target of a trans-acting SAM riboswitch. PrfA is essential for PrfA virulence. Addition of a specific low molecular weight compound block bacterial invasion through the PrfA pathway. The PrfA protein is not essential for viability, but absence of *prfA* attenuates *Listeria monocytogenes* virulence. The PrfA protein structure has previously been determined for PrfA by us and by partner P3 (Velge *et al.*, Microbiology 153:995 - 1005. 2007). Addition of a certain small molecule to *L. monocytogenes* (class of 2-pyridones) blocks its invasive capacity as determined by cell-invasion experiments using Hep-2 cells. Importantly, the small molecule does not affect the viability of the bacteria or the eukaryotic cells. Subsequent experiments using various techniques suggest this small molecule to specifically target the PrfA pathway or the activity/level of *prfA*/PrfA itself.

2 DISSEMINATION AND USE

2.1 Exploitation of Results

Exploitable knowledge	Exploitable measure(s)	Sector(s) of application	Timetable for use	IPR protection	Owner
Methodologies for genome-wide search for novel ncRNAs	Methods	Industrial	2007 2008	patent is possible in 2007, 2008	P1, P6
Final situation			**2007, 2008**	**published**	**P1, P6**
Methodologies for analysis of ncRNAs their targets and regulatory mechanisms	Methods	Industrial	2007 2008	patent is possible in 2007, 2008	P1, P2, P3, P4
Final situation			**2007, 2008**	**published**	**P1, P2, P3, P4**
ncRNAs data in bacteria /pathogenic bacteria, ncRNA targets	Method Small molecules	Industrial	2007 2008 2009	patent is possible in 2007, 2008	P1, P2, P3, P4, P5, P6
Final situation			**2009 time not yet clear**	**to publish patent is possible**	**P1, P2, P3, P4, P5, P6**
Novel targets for therapeutic intervention	Method Small molecules	Industrial	2009	patent is possible in 2009	P1, P2, P3, P4, P5, P6
Final situation			**time not yet clear**	**patent is possible**	**P1, P3, P4, P5, P6**

Table 1

2.1.1 Brief description of Exploitable Results

For the moment, no patents have been deposited, because the validation of targets needs further work. The possibility for detecting essential targets is still open and very probable. The discovery of small molecules that interfere with virulence is also still a goal for the future. We will continue and put all our efforts in realizing these goals.

Even though the consortium did not succeed to raise interest of companies (SMEs) in giving a talk or participating at the EMBO workshop, we are very confident that the advanced state of the art of our work will increase the interest of companies (SME). We will ask them to participate in our future projects (COST and others).

Methods were published not patented due to a lack of commercial potential.

2.2 Dissemination of Knowledge

Type	Type of audience	Countries addressed	Partner(s) responsible	Actual Date*
Project website	General public	Austria, France Sweden, Israel	P8 / all	02/06
Project website update	General public	Austria, France, Sweden, Israel	P8 / all	Latest version 03/09
Press releases (press/radio/TV)	General public	Austria, France Sweden, Israel	P8 / all	02/06, 05/08, 02/09, 06/09
Websites of partners' institution	Higher education	Austria, France, Sweden, Israel	P8 / all	02/06
Scientific workshops	Young researchers	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6, P8	04/06,10/06,05/07,07/07,01/08,09/08
Lab training	Young researchers	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6	2006-2009
Scientific publications	Researcher, Industry	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6	2006-2009
Posters	Researcher	Austria, France, Sweden, Israel, USA, others to be defined	P1, P2, P3, P4, P5, P6	2006-2009
Conferences	Researcher, Industry	Austria, France, Sweden, Israel, USA, others to be defined	P1, P2, P3, P4, P5, P6	2006-2009
Lectures	Higher education	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6	2006-2009
Seminars	Researcher	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6	2006-2009
Symposium	Researcher, SME	Austria, France, Sweden, Israel	P1, P2, P3, P4, P5, P6	01/09
Public lecture	General public	Austria	P1	None on the topic.

Table 2 *month/year

2.2.1 Brief Description of Major Activities

The consortium vividly transferred the new knowledge about the role of non-coding RNAs in bacterial pathogenicity in talks and posters to the scientific community at numerous international conferences. They were invited for various talks in seminars and lectures at national and international institutions and meetings.

The plan was checked regularly for changes and adaptations but kept the same during the lifetime of the project.

Although no patents could be submitted many protocols, methods and new insights into the project topic were published in 56 papers (list below) in journals with a high impact factor during the lifetime of BACRNAs. 8 papers were jointly published.

The consortium published a description of the project BACRNAs in the EC funded book „ Fundamental Genomics Book", 2008. It also published "Searching for novel targets to fight bacterial infections" in the journal eStrategies 2009. The consortium is also about to create a review about the role of non-coding RNAs in bacterial pathogenicity. Further press releases and publications, which are suitable for a broader public were not published since appropriate results were not available.

However, the partners were involved in the organisation and co-organisation of frequent meetings, workshops and conferences. With the EMBO workshop on „ New Function of Regulatory RNAs in Pro- & Eucaryotes" we succeed to create and organise a very successful closure of BACRNAs and in the same time opening of a discussion platform for future projects.

2.3 Publication list for the full duration of the project

Joint Publications

Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Loh E, Gripenland J, Barthelemy M, Vergassola M, Nahori MA, Soubigou G, Régnault B, Coppée JY, Lecuit M, **Johansson** J, **Cossart** P. (2008). The transcriptional landscape of *Listeria monocytogenes*: switch from saprophytism life to virulence. *Manuscript submitted*.

Romby P, **Wagner** EGH. (2008) Exploring the complex world of RNA regulation. *Biol. Cell* 100, e1-e3.

Padalon G. Hershberg R. Elgrably-Weiss M. Baruch K. Rosenshine I. **Margalit** H. and **Altuvia** S. (2008) Small RNAs enc ded within genetic islands of Salmonella typhimurium show host-induced expression and role in virulence. *Nuc. Acid. Res.* 36: 1913-1927.

Shimoni Y. Friedlander G. Hetzroni G. Niv G. **Altuvia** S. Biham O. **Margalit** H. (2007) Regulation of gene expression by small non-coding RNAs: a quantitative view. *Mol Syst Biol*. 3: 138.

Charpentier E, **Schroeder** R. (2007) RNA techniques for bacteria. *Curr Opin. Microbiol*., 8, 254-6

Mandin P, Repoila F, Vergassola M, **Geissmann** T, **Cossart** P. (2007) Identification of new noncoding RNAs in Listeria monocytogenes and prediction of mRNA targets. *Nucleic Acids Res.* 35(3), 962-974

Velge P, Herler M, **Johansson J**, Roche SM, Témoin S, Fedorov AA, Gracieux P, Almo SC, Goebel W, **Cossart** P. (2007) A naturally occurring mutation K220T in the pleiotropic acti-

vator PrfA of *Listeria monocytogenes* results in a loss of virulence due to decreasing DNA-binding affinity. *Microbiology* 153:995 - 1005.

Romby P, Vandenesch F, **Wagner** EGH. (2006) The role of RNAs in the regulation of virulence gene expression. *Current in Opinion in Microbiology* 9(2), 229-36.

2009

Simonetti A, Marzi S, Jenner L, Myasnikov A, **Romby** P, Yusupova G, Klaholz BP, Yusupov M. (2009) A structural view of translation initiation in bacteria. *Cell Mol Life Sci.* 66, 423-436.

Chevalier C, Geissmann T, Helfer AC, **Romby** P. (2009) Probing mRNA structure and sRNA-mRNA interactions in bacteria using enzymes and lead (II). In "*Methods in Molecular Biology*", (ed. Serganov A), Vol 540, Humana Press.

Fechter P, Chevalier C, Yusupova G, Yusupov M, **Romby** P, Marzi S. (2009) Ribosomal initiation complexes probed by toeprinting and effect of trans-acting translational regulators in bacteria. In "*Methods in Molecular Biology*", (ed. Serganov A), Vol 540, Humana Press.

Geissmann T, Marzi S, **Romby** P. (2009) The role of mRNA structure in translational control in bacteria. *RNA Biol.* In press.

2008

Vecerek B, Rajkowitsch L, Sonnleitner E, **Schroeder** R, Blasi U. (2008) The C-terminal domain of Escherichia coli is required for regulation. *Nucleic Acids Res,* 36, 133-143

Windbichler N, von Pelchrzim F, Rajkowitsch L, Mayer O, Cazsar E, **Schroeder** R. (2008) Isolation of ncRNA-binding proteins from *E. coli* : evidence for frequent binding of RNAs to RNA polymerase. *RNA Biol*, **5**, 30-40

Siller M, Janapatla RP, Pirzada ZA, Hassler C, Zinkl D, **Charpentier** E. (2008) Functional analysis of the group A streptococcal luxS/AI-2 system in metabolism, adaptation to stress and interaction with host cells. *BMC Microbiology*. 8:188.

Gratz N, Siller M, Schaljo B, Pirzada ZA, Gattermeier I, Vojtek I, Kirschning CJ, Wagner H, Akira S, **Charpentier** E, Kovarik P. (2008) Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9. *J. Biol. Chem*. 283(29):19879-19887.

Vojtek I, Pirzada ZA, Henriques-Normark B, Mastny M, Janapatla RP, **Charpentier** E. (2008) Lysogenic transfer of group A Streptococcus superantigen gene among Streptococci. *J. Infect. Dis*. 197(2):225-234.

Sonnleitner E, Sorger-Domenigg T, Madej MJ, Findeiß S, Hackermüller J, Hüttenhofer A, Stadler P, Bläsi U, **Moll** I. (2008) Detection of small non-coding RNAs in Pseudomonas aeruginosa by RNomics and structure-based bioinformatic tools. *Microbiology*, 154, 3175-87.

Marzi S, Fechter P, Chevalier C, **Romby** P, Geissmann T. (2008) RNA switches regulate initiation of translation in bacteria. *Biol. Chem*. 389 : 585-598.

Chevalier C, Huntzinger E, Fechter P, Boisset S, Vandenesch F, **Romby** P & Geissmann T (2008) Staphylococcus aureus endoribonuclease III : purification and properties. *Methods in Enzymology* 447: 309-327.

Beckert B, Nielsen H, Einvik C, Johansen SD, **Westhof** E, Masquida B. (2008) Molecular modelling of the GIR1 branching ribozyme gives new insight into evolution of structurally related ribozymes. *EMBO J.* 27: 667-678.

Bioinformatic program and web server : PARADISE (Platform to analyze RNA annotations over a distributed environment) (http://paradise-ibmc.u-strasbg.fr/bin/view/Main/WebHome). F. Jossinet & E. **Westhof**

Unoson C, **Wagner** EGH. (2008) A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli. *Mol. Microbiol.* 70, 258-270.

Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, **Johansson** J, Vogel J, Wai SN. (2008) A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Molecular Microbiology* 70:100-111.

2007

Altuvia S. Identification of bacterial small non-coding RNAs: experimental approaches. *Curr Opin Microbiol*. 10: 257-261. 2007.

Weinstein-Fischer D, **Altuvia** S. (2007) Differential regulation of Escherichia coli topoisomerase I by Fis. *Mol Microbiol*. 63(4), 1131-1144.

Heidrich N, **Moll** I, Brantl S. (2007) In vitro analysis of the interaction between the small RNA SR1 and its primary target ahrC-RNA. *NAR*, 35(13), 4331-46.

Marzi S, Myasnikov AG, Serganov A, Ehresmann C, **Romby** P, Yusupov M, Klaholz BP. Structured mRNAs regulate translation initiation by binding to the platform of the ribosome. *Cell*. 2007 Sep 21;130(6):1019-31.

Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M, Chevalier C, Helfer AC Benito Y, Jacquier A, Gaspin C, Vandenesch F, **Romby** P. (2007) Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*. 21(11), 1353-1366.

Romby P, Springer M. (2007) Translational Control in Prokaryotes. In Translational Control in Biology and Medicine, (Hershey J, Sonnenberg N, Metthews M, eds.), *Cold Spring Harbor Laboratory Press*, pp 807-832.

Toledo-Arana A, Repoila F, **Cossart** P. (2007) Small noncoding RNAs controlling pathogenesis. *Curr Opin Microbiol* 10:182-188

Udekwu KI, **Wagner** EGH. (2007) The Escherichia coli luxS mRNA is processed in an RNase III-dependent fashion in response to MicA RNA accumulation. In: *thesis Klas Udekwu*, *ISSN* 1651-6214.

Udekwu K, **Wagner** EGH. (2007) Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res*. 35, 1279-1288.

Ivanova N, Lindell M, Pavlov M, Holmberg-Schiavone L, **Wagner** EGH, Ehrenberg M. (2007) Structure probing of tmRNA in distinct stages of trans-translation and its interaction with SmpB. *RNA* 13, 713-22.

Gerdes K, **Wagner** EGH. (2007) RNA Antitoxins. *Curr. Opin. Microbiol.* 10, 117-124.

Vogel J, **Wagner** EGH. (2007) Target identification of small non-coding RNAs in bacteria. *Curr. Opin. Microbiol.* 10, 262-270.

Darfeuille F, Unoson C, Vogel J, **Wagner** EGH. (2007) An antisense RNA inhibits translation by competing with "standby" ribosomes. *Mol. Cell* 26, 381-392.

Hinas A, Reimegård J, **Wagner** EGH, Nellen, W., Ambros, V. & Söderbom, F. (2007) The small RNA repertoire of Dictyostelium discoideum: small antisense RNAs that may be derived

from longer transcripts and multiple classes of repeat-associated RNAs. *Nucleic Acids Res.* 35, 6714-6726.

Unoson C, **Wagner** EGH. (2007) Dealing with stable structures at ribosome binding sites: bacterial translation and ribosome standby. *RNA Biol.* 4, e1-e6.

Hammann C, **Westhof** E. (2007) Searching genomes for ribozymes and riboswitches. *Genome Biol.* 8, 210

2006

Elgrably-Weiss M, Schlosser-Silverman E, Rosenshine I, **Altuvia** S. (2006) DeoT, a deoR-type transcriptional regulator of multiple target genes. *FEMS Microbiol Lett.* 254, 141-148.

Geissmann T, Possedko M, Fechter P, Huntzinger E, Ehresmann C, **Romby** P. (2006) Regulatory RNAs as mediator of gene expression in bacteria. *Handb Exp Pharmacol.* 173, 9-43.

Lorenz C, von Pelchrzim F, **Schroeder** R. (2006) Genomic systematic evolution of ligands by exponential enrichment (Genomic SELEX) for the identification of protein-binding RNAs independent of their expression lebels. *Nature Protocols*, 1(5), 2204-2212.

Prenninger S, **Schroeder** R, Semrad K. (2006) Assaying RNA chaperone activity in vivo in bacteria using a ribozyme folding trap. *Nature Protocols,* 1(2), 1274-1278.

Piganeau N, **Schroeder** R. (2006) Identification and detection of RNA-RNA interactions using the yeast RNA hybrid system. *Nature Protocols*, 1(2), 689-694.

Winbichler N, **Schroeder** R. (2006) Isolation of specific RNA-binding proteins using the streptomycin-binding RNA aptamer. *Nature Protocols*, 1(2), 637-640.

Piganeau N, Schauer U, **Schroeder** R. (2006) A yeast RNA-hybrid system for the detection of RNA-RNA interactions in vivo. *RNA,* 12, 177-84.

Lorenz C, **Schroeder** R. (2006) Aptamers to Antibiotics. In "*The Aptamer Handbook*", Ed: Sven Kussmann, WILEY-VCH GmbH & Co. KgaA, Weinheim, pp 116-130.

Dangerfield JA, Winbichler N, Salmons B, Guenzburg WH, **Schroeder** R. (2006) Enhancement of the StreptoTag method for isolation of low abundance proteins with complex RNA binding targets. *Electrophoresis*, 27(10):1874-7.

Lorenz C, Piganeau N, **Schroeder** R. (2006) Stabilities of HIV-1 DIS type RNA loop-loop interactions in vitro and in vivo. *Nucleic Acids Res*. 34, 334-42.

Wagner EGH, Darfeuille F. (2006) Small regulatory RNAs in bacteria. In: *Small RNAs: Analysis and regulatory functions* (eds. Nellen W, Hammann C.), Springer Verlag, Germany, pp. 1-29.

Leontis NB, Altman RB, Berman HM, Brenner SE, Brown JW, Engelke DR, Harvey SC, Holbrook SR, Jossinet F, Lewis SE, Major F, Mathews DH, Richardson JS, Williamson JR, **Westhof** E. (2006) The RNA Onthology Consoirtium: an open invitation to the RNA community. *RNA*. 12(4), 533-541.

Leontis NB, Lescoute A, **Westhof** E. (2006) The building blocks and motifs of RNA architecture. *Curr Opin Struct Biol*. 16(3), 279-287.

Kondo J, Urzhumtsev A, **Westhof** E. (2006) Two conformational states in the crystal structure of the Homo sapiens cytoplasmic ribosomal decoding A site. *Nucleic Acids Res*. 34(2):676-85.

Moitessier N, **Westhof** E, Hanessian S. (2006) Docking of aminoglycosides to hydrated and flexible RNA. *J Med Chem*. 49(3):1023-33.

Lescoute A, **Westhof** E. (2006) Topology of three-way junctions in folded RNAs. *RNA*. 12(1):83-93.

3 PUBLISHABLE EXPLOITABLE RESULTS

For the moment, no patents have been deposited, because the validation of targets needs further work. The possibility for detecting essential targets is still open and very probable and the discovery of small molecules that interfere with virulence is also still a goal for the future. We will continue and put all our efforts in realizing these goals.