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Global Discovery of Small Noncoding RNAs in Pathogenic *Yersinia* Species

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38.1 Introduction

Small noncoding RNAs (sRNAs) function as regulatory elements in both eukaryotes and bacteria. *Trans*-acting bacterial sRNAs posttranscriptionally regulate gene expression by base pairing with target mRNAs, which often leads to changes in translation efficiency and/or stability of the transcript. Bioinformatic search algorithms along with a variety of experimental approaches have become increasingly useful for the discovery of sRNAs and their mRNA targets. Our laboratory and others recently demonstrated that Hfq, a protein chaperone of sRNAs in bacteria, is required for the full virulence of both *Yersinia pestis*, the bacterium that causes the disease plague, and the genetically related gastrointestinal pathogen *Yersinia pseudotuberculosis*. This led us to pursue the first global identification and analysis of sRNAs in pathogenic *Yersinia* species. We have identified 150 previously unannotated sRNAs expressed by *Y. pseudotuberculosis* when cultured in vitro at either 26°C or 37°C, the majority of which are *Yersinia*-specific. The deletion of multiple *Yersinia*-specific sRNAs from either *Y. pseudotuberculosis* or *Y. pestis* leads to the attenuation of these pathogens in mouse models of infection. In addition, we have identified the mRNA targets controlled by one of these virulence-associated sRNAs, suggesting potential new virulence determinants in *Y. pseudotuberculosis*.

38.2 Bacterial Small RNAs (sRNAs): An Introduction

In recent years it has become clear that proteins are not the only modulators of gene expression. Small noncoding RNAs (sRNAs) in both bacteria and eukaryotes are now recognized as major components of diverse regulatory circuits. Bacterial sRNAs are heterogeneous in size (most are 50–500 nucleotides long) and are typically encoded in intergenic regions (IGRs). They are independently transcribed from their own promoters, contain ρ -independent terminators, and are usually not processed (Waters and Storz 2009). The majority of sRNAs control gene expression at the posttranscriptional level by base pairing within the 5' untranslated region (UTR) of their target mRNAs. This RNA–RNA interaction leads to alterations in mRNA target translation or half-life (Gottesman and Storz 2010; Sharma and Vogel 2009). The predominant outcome of the sRNA–target mRNA interaction is the downregulation of gene expression (Sharma et al. 2007; Urbanowski et al. 2000), but positive regulation by sRNAs has also been described (Tramonti et al. 2008; Vogel and Papenfort 2006). The sRNA contact on the mRNA target is typically short (6–8 contiguous base pairs) and imperfect in part because the sRNA is encoded *in trans* in a distal

genomic location, and in most cases requires the RNA chaperone protein Hfq to presumably stabilize the sRNA–mRNA interaction (McCullen et al. 2010).

38.2.1 Approaches for the Global Discovery of sRNAs

The exact number of sRNAs encoded in the genomes of most bacteria is still not known, although hundreds of sRNAs have recently been discovered in dozens of bacterial species. The identification of sRNAs has been challenging due to the unique features of these RNAs: (1) they are relatively small in size and this makes them resistant to single nucleotide mutagenesis; (2) they typically do not encode proteins and thus cannot be identified by simple searches for open reading frames; (3) the primary sequence of sRNAs is conserved only between closely related bacterial species; and (4) they have been omitted from many genetic screens, such as those using transposon mutagenesis, because they are encoded in the IGRs. The earliest studies relied on computational methods involving homology searches within the IGRs of closely related bacterial species and included the prediction of σ^{70} promoters and transcription terminators (Livny and Waldor 2007). More recently the use of bioinformatic algorithms that do not rely on primary sequence conservation as a predictive criterion has discovered additional potential sRNAs within the genomes of numerous bacterial species (Livny et al. 2006). However, the majority of the sRNAs identified by this method still warrant experimental validation.

In addition to the evolution of biocomputational means for sRNA discovery, there has recently been an explosion of experimental approaches for genome-wide detection of expressed sRNAs. These methodologies include the use of DNA microarrays, RNA-sequencing (RNA-Seq), and co-immunoprecipitation with sRNA-binding proteins (Vogel and Sharma 2005). High-density (tiling) microarrays, which cover both strands of the genome and include the IGRs, have successfully been used for global discovery of sRNAs in *Caulobacter crescentus* (Landt et al. 2008), *Listeria monocytogenes* (Toledo-Arana et al. 2009), *Mycobacterium leprae* (Akama et al. 2009), and *Streptococcus pneumoniae* (Kumar et al. 2010). The low-density arrays spotted with oligonucleotides or PCR fragments containing a defined set of regions of a particular genome have been useful in validating predicted sRNAs, and examples of these include studies of pathogenesis-relevant sRNAs in *Staphylococcus aureus* (Pichon and Felden 2005) and the sporulation network of *Bacillus subtilis* (Silvaggi et al. 2006).

With the advances in high-throughput sequencing techniques, RNA-Seq has been the leading approach for global transcriptomic analysis and sRNA discovery in bacteria. Currently available technologies include 454 pyrosequencing, SOLEXA, and SOLiD, and have all been applied to the identification of new sRNAs (MacLean et al. 2009; Srivatsan et al. 2008). Transcriptome analysis of *Burkholderia cenocepacia* strains grown under specific environmental conditions using the Illumina-SOLEXA platform resulted in the identification of thirteen sRNAs (Yoder-Himes et al. 2009). The SOLiD platform has been compared to SOLEXA in the transcriptomic profiling of *B. anthracis* and deemed suitable for sRNA discovery (Passalacqua et al. 2009), while Liu et al. applied the 454 method to *Vibrio cholerae*, which yielded hundreds of candidate sRNAs (Liu et al. 2009). Most recently, differential RNA-Seq, which is selective for the 5' end of primary transcripts, has been employed by Sharma et al. to generate the operon map in *Helicobacter pylori* and has concomitantly allowed for the discovery of 60 previously unidentified sRNAs (Sharma et al. 2010). This approach has also been used in the GC-rich Gram-positive *Streptomyces coelicolor* and has resulted in the identification of 63 sRNAs, the majority of which are growth phase-dependent for their expression (Vockenhuber et al. 2011).

Lastly, sRNAs have been identified by co-purification with proteins. The sRNA chaperone protein Hfq has most commonly served as bait in these enrichment experiments, including

one of the original global studies of sRNAs in *E. coli* in which interacting sRNAs were identified by co-immunoprecipitation with Hfq followed by tiling microarray hybridization (Zhang et al. 2003). Similar approaches have been successfully used in *L. monocytogenes* (Christiansen et al. 2004) and *Pseudomonas aeruginosa* (Sonnleitner et al. 2008). Sittka et al. combined co-immunoprecipitation of sRNAs using a chromosomally encoded, FLAG-tagged Hfq in *Salmonella* with RNA-Seq to identify not only Hfq-associated sRNAs but also potential mRNA targets (Sittka et al. 2008).

38.2.2 Approaches for sRNA Target Identification and Validation

To fully understand the biological function of a sRNA, identification of the cognate interacting mRNA target is required. Since it is now recognized that many sRNAs regulate multiple targets, a diverse set of tools are available for the genomewide discovery of targets. Several biocomputational approaches, including the programs TargetRNA (Tjaden 2008) and IntaRNA (Busch et al. 2008), have been developed to predict the mRNA targets of sRNAs based on the short and imperfect complementarity required for interaction. “Wet lab” experimental tools, including microarrays and proteomics, rely on the fact that the target regulation results in changes in mRNA and/or protein levels. These approaches are typically coupled to overexpression of sRNAs from a strong promoter or in sRNA-deletion backgrounds. For instance, pulse expression of sRNAs from the tightly controlled, arabinose-inducible P_{BAD} promoter followed by microarray analysis revealed 18 potential targets for the iron starvation regulator RyhB sRNA of *E. coli* (Masse et al. 2005). A similar approach resulted in the identification of targets for the *Salmonella* RybB and *E. coli* OmrAB sRNAs that regulate outer membrane protein-encoding mRNAs (Guillier and Gottesman 2006; Papenfort et al. 2006). Proteomic analysis of strains lacking or overexpressing single sRNAs identified *ompA* as a MicA target (Rasmussen et al. 2009) and overexpression of the sRNA Spot42 led to a specific decrease in GalK protein levels (Moller et al. 2002). Once the mRNA targets of an sRNA are identified, regulation by the sRNA is usually verified by either chromosomal or plasmid-based mRNA fusions to reporter genes (Mandin and Gottesman 2009; Urban and Vogel 2007) or by direct assessment of target protein levels by immunoblot (or a chromosomal epitope tagging if no specific antibody is available (Koo et al. 2011).

The approaches presented here each have advantages and limitations. Bioinformatic analyses rely on the base-pairing interaction of the sRNA with its mRNA target, which implies complementarity. While this has been the major criterion for target identification using a bioinformatic approach, some of the algorithms that have been developed to identify previously uncharacterized sRNA–mRNA interactions have occasionally failed to detect known pairings. A major hurdle in developing computational methods is our incomplete understanding of the rules that govern the imperfect sRNA–mRNA interactions and the physical constraints that may be involved. The use of microarrays for target identification has been very successful due to the availability of whole genome cDNA and tiling microarrays for many bacterial species. This method, however, makes assumptions about the mechanisms by which the sRNA in question controls its target, as it is biased toward regulatory mechanisms that affect transcript levels. In some cases, sRNAs may alter only target translation and thus would be omitted in this type of analysis. Unfortunately, both microarray and proteomic strategies for target identification are limited by the inability to distinguish between direct and secondary targets. Proteomic analysis, however, has an advantage in that it can determine whether protein abundance is affected by a sRNA that regulates translation without causing a change in transcript level.

38.2.3 sRNA-Mediated Control of Virulence

While the specific functions of many sRNAs are still unknown, it is clear that sRNAs act to integrate extracellular signals that aid bacteria in adjusting to the environment and in the response to a variety of stresses. The control of virulence determinants important for bacterial infections is also coordinated by sRNAs. This includes mechanisms of direct sRNA–mRNA pairing and also through the binding of sRNAs to proteins. The end result of these interactions is the fine tuning of the metabolic requirements of pathogenic bacteria to endure the stress imposed by the host as well as the expression of virulence factors.

For example, a study of *S. typhimurium*-specific genetic islands led to the identification of 28 candidate sRNAs (Padalon-Brauch et al. 2008). Several of these sRNAs are induced when *Salmonella* resides within macrophages and one sRNA, IsrJ, was found to affect the translocation efficiency of virulence-associated effector proteins into nonphagocytic cells (Padalon-Brauch et al. 2008).

In *S. aureus*, RNAIII can both positively and negatively regulate targets that are involved in virulence. RNAIII pairs with the 5' UTR of the hemolysin gene *hla* and destabilizes the secondary stem-loop structure that sequesters the ribosome-binding site to activate the translation of the hemolysin (Morfeldt et al. 1995). The same sRNA negatively regulates the synthesis of an adhesin encoded by the *spa* gene (Huntzinger et al. 2005) as well as the transcriptional regulator RotA (Geisinger et al. 2006). In addition to RNAIII, there are three other sRNAs located on the pathogenicity island of *S. aureus* that may also be involved in regulation of virulence determinants (Pichon and Felden 2005).

More recent global studies of sRNAs in bacterial pathogens have identified the 6S RNA as a required factor for the optimal intracellular replication of *Legionella pneumophila* (Faucher et al. 2010) and several sRNAs that allow *M. tuberculosis* to respond to various stress conditions (Arnvig and Young 2009). Also, Ramirez-Pena et al. have shown that the FasX sRNA in *Streptococcus* positively regulates the expression of the virulence factor streptokinase (Ramirez-Pena et al. 2010), while the iron-regulated sRNA RyhB regulates pathogenesis of *Shigella dysenteriae* (Murphy and Payne 2007).

38.2.4 Noncoding RNAs of *Yersinia*

At the time we initiated the studies described in this report, there were only 15 sRNAs annotated in the genome of *Y. pseudotuberculosis* strain IP32953 and 7 sRNAs annotated for *Y. pestis* strain CO92. A computational analysis by Livny et al. predicted that ~1,400 sRNAs may be encoded within the genome of *Y. pestis* (Livny et al. 2006), although no global experimental examination of sRNAs in *Yersinia* had yet been attempted.

In 2003, Delihias predicted the presence of MicF, an sRNA that regulates OmpF in *E. coli*, within the *Y. pestis* and *Y. enterocolitica* genomes. He determined that MicF of *Y. pestis* shares ~53% conservation with the *E. coli* ortholog, and also identified additional sequence differences between the *Y. pestis* and *Y. enterocolitica* sRNAs (Delihias 2003).

More recently, Horler and Vanderpool identified SgrS, an sRNA that regulates the metabolic stress response, in the genomes of both *Y. pestis* and *Y. pseudotuberculosis* (Horler and Vanderpool 2009). Unlike in other Enterobacteriaceae, SgrS in *Yersinia* is not predicted to encode the small peptide SgrT that facilitates target interaction. Based on these studies, the authors hypothesized that the predicted target-interacting region in the SgrS of *Y. pestis* and *Y. pseudotuberculosis* is longer than in other closely related species to presumably facilitate a more stable interaction with the mRNA target. Additionally, Wadler and Vanderpool showed that the *Y. pestis* SgrS can rescue an *E. coli* SgrS mutant in base-pairing function, but the lack of SgrT in *Y. pestis* cannot complement the translation defect of the *E. coli* SgrS

mutant (Wadler and Vanderpool 2009). Therefore, the absence of SgrT in *Y. pestis* suggests that the plague pathogen may not require this peptide to respond to stress.

The Csr system, including the noncoding RNAs CsrB and CsrC and their cognate RNA-binding protein CsrA, has also been described in *Y. pseudotuberculosis*. Heroven et al. determined that this posttranscriptional regulatory system is a part of a global pathway that allows *Yersinia* to adapt to metabolic and environmental stresses (Heroven et al. 2008). The authors found that the Csr system affects the global virulence gene regulator RovA by controlling the synthesis of the LysR-type regulator RovM. The components of the Csr system in *Y. pseudotuberculosis* appear to be differentially regulated in response to a variety of growth conditions and, much like in other bacterial pathogens, this system plays a role in the host–pathogen interaction.

The unique RNA molecule SsrA, which functions as both a tRNA and an mRNA encoding a short peptide tag, and its chaperone protein SmpB are highly conserved and participate in the quality control of translation (Karzai et al. 2000). Recent studies have identified the SsrA–SmpB system to be critical for the pathogenesis of both *Y. pseudotuberculosis* (Okan et al. 2006) and *Y. pestis* (Okan et al. 2010). The attenuation of the *ssrA–smpB* mutants in both *Yersinia* species is associated with the reduction in the synthesis and secretion of type III secreted proteins and Okan et al. have presented evidence that immunization of mice with *ssrA–smpB* deletion strains of *Y. pestis* leads to protection against a subsequent lethal intranasal challenge with fully virulent *Y. pestis* (Okan et al. 2010).

Finally, it has been established that the GlmY/GlmZ sRNAs contribute to the regulation of the GlmS enzyme. A recent study determined that while the regulation of GlmZ and GlmY transcription in *E. coli* is achieved through a σ^{70} promoter, in *Y. pseudotuberculosis* σ^{54} promoters regulate expression of the sRNA (Gopel et al. 2011). The significance of this regulatory difference is not yet understood.

38.2.5 The Small RNA Chaperone Hfq of *Yersinia*

In most bacteria, canonical *trans*-acting sRNAs require the chaperone Hfq to mediate and enhance the limited base-pairing interaction with their mRNA targets. In *Yersinia*, the gene for Hfq was discovered in a screen for regulators of the heat-stable toxin Yst of *Y. enterocolitica* and was designated as *yjp* (Nakao et al. 1995). Deletion of Hfq in *Y. enterocolitica* and many other bacterial species has pleiotropic effects (Meibom et al. 2009; Nakao et al. 1995) and it has been shown that Hfq plays a role in the virulence in a number of bacterial pathogens (Christiansen et al. 2004; Fantappie et al. 2009; Kulesus et al. 2008). Recent work from our laboratory has established that Hfq is critical for the pathogenesis of *Y. pseudotuberculosis* in the mouse model of Yersiniosis and affects motility, type III secretion, and intracellular survival (Schiano et al. 2010). Additionally, Geng et al. determined that Hfq is required for the full virulence of *Y. pestis* in the intravenous and subcutaneous models of mouse infection (Geng et al. 2009). This loss of virulence may be due to impaired replication and/or persistence of bacteria within the host macrophages, especially during the initial stage of infection (Geng et al. 2009). This suggests that Hfq, together with the sRNAs it controls, regulates essential virulence determinants in *Yersinia*.

38.3 Global Identification of sRNAs Expressed by *Y. pseudotuberculosis*

As Hfq is required for the full virulence of *Yersinia* species (and therefore, by association, sRNAs), the goal of our study was to identify all sRNAs expressed by *Y. pseudotuberculosis* in an unbiased fashion. For this purpose we performed Illumina-SOLEXA-based deep sequencing on sRNA libraries generated from *Y. pseudotuberculosis* IP32953 grown under multiple conditions (Koo et al. 2011). Our deep sequencing analysis resulted in ~2.5–17

million 36 nt long reads which were categorized into different RNA species. The RNAs corresponding to IGRs were subsequently clustered and analyzed for conserved features such as promoters and ρ -independent terminators, yielding a list of 165 potential sRNAs. This analysis confirmed the expression of the 15 previously annotated regulatory RNAs in the *Y. pseudotuberculosis* genome and identified 150 previously unannotated sRNAs. This method proved to be extremely sensitive in that it uncovered RNAs whose levels are not detectable by Northern blot (Koo et al. 2011). We refer to the sRNAs we identified in this study as Ysrs (for *Yersinia* small RNAs).

BlastN analysis of Ysrs determined that 32 sRNAs encoded in the *Y. pseudotuberculosis* genome are represented by orthologous sequences in the *E. coli* and *S. typhimurium* genomes (Fig. 38.1, light gray) and these include many previously characterized sRNAs such as MicA, FnrS/Stn520, RprA, GcvB, RybB, RhyB, GlmY, GlmZ, and OmrA/B (Coornaert et al. 2010). On the other hand, 75% of the Ysrs we identified are specific to *Y. pseudotuberculosis* and *Y. pestis* in that they do not show sequence conservation with other bacterial species (Fig. 38.1, dark gray). In addition, we identified 6 Ysrs that are *Y. pseudotuberculosis*-specific (for which there are no homologous sequences in the genome of *Y. pestis*) (Fig. 38.1, black). The *Yersinia*-specific sRNA group contains 63 Ysrs encoded by both *Y. pseudotuberculosis* and *Y. pestis* with single or multiple differences in sequence between the species (i.e. mismatches, deletions, insertions - Fig. 38.1, right panel). These may be significant in that a single nucleotide mismatch between an sRNA and its target can abolish or alter the regulatory effect. Additionally, Northern blot analysis revealed a difference in timing, temperature, and Hfq requirement for the expression of a subset of RNAs that are conserved between *Y. pestis* and *Y. pseudotuberculosis* (Koo et al. 2011). This suggests that evolutionary changes in posttranscriptional regulation between these species have led to a distinct temporal regulation of potentially conserved target mRNAs (including virulence determinants).

38.4 Contribution of Newly Identified *Yersinia* sRNAs to Virulence

To determine if any of the sRNAs we identified by deep sequencing contribute to the virulence of *Y. pseudotuberculosis*, we generated bacterial mutants deleted for the sRNAs Ysr29, a *Y. pseudotuberculosis*-specific sRNA, Ysr35, which is conserved between *Y. pestis* and *Y. pseudotuberculosis*, and RybB, a sRNA found in many Gram-negative bacteria. While the deletions of these RNAs did not affect bacterial growth or the expression of neighboring genes (Koo et al. 2011), deletions of Ysr29 and Ysr35 resulted in a significant attenuation of bacteria in a mouse model of Yersiniosis (Fig. 38.2a). These results implicate Ysr29 and Ysr35 as potential regulators of virulence determinants. On the other hand, deletion of RybB did not significantly affect the virulence of *Y. pseudotuberculosis*.

In addition, we found that the deletion of Ysr35 from the genome of *Y. pestis* also attenuated the pathogen in a mouse model of pneumonic plague (Fig. 38.2b). This suggests that *Y. pseudotuberculosis* and *Y. pestis* encode at least one conserved RNA that controls virulence, but it is not yet known whether the targets of this sRNA are also conserved between the two species, or whether *Y. pestis* has acquired and/or lost targets specifically regulated by this RNA.

38.5 Proteomic Analysis for Target Identification

Considering the uniqueness of Ysr29 to *Y. pseudotuberculosis* and its contribution to virulence, we performed a proteomic analysis using 2D differential gel electrophoresis (2D-DIGE) to determine the regulated targets of this sRNA. We compared protein profiles from whole-cell lysates of wild-type *Y. pseudotuberculosis* to those of the Δ Ysr29 strain grown to stationary phase at 26°C, the time point at which this sRNA is most abundant. A

comparison of protein profiles between the wild-type and Δ Ysr29 strains showed 16 spots with 1.5-fold or more difference in fluorescence intensity (Fig. 38.3) and identified 8 proteins regulated by Ysr29 using MALDI-TOF mass spectroscopy (Table 38.1). Significantly, each of these potential targets could be involved in virulence since all are required for the proper response of bacteria to a variety of stresses (Allocati et al. 2009; Rowley et al. 2006).

We verified the effects of Ysr29 at the posttranscriptional level by generating chromosomal in-frame fusions of the GST, RpsA, OmpA, and GroEL coding regions with the HA-epitope tag in both wild-type and Δ Ysr29 strains. Levels of fusion proteins were measured by western blot analysis using an anti-HA antibody and we confirmed that GST is more abundant in the Δ Ysr29 strain than in the wild-type background, while RpsA, OmpA, and GroEL are elevated in the wild type as compared to the Δ Ysr29 strain, demonstrating posttranscriptional regulation by this sRNA (Koo et al. 2011).

38.6 Conclusions

Small noncoding RNAs have been recognized as critical regulators of gene expression in bacteria. In recent years there has been an abundance of studies that have used global approaches to discover sRNAs and their mRNA targets in many bacterial species. Prior to our work, however, knowledge about *Yersinia* sRNAs has been limited. By using RNA-Seq, we have identified the global set of sRNAs expressed by *Y. pseudotuberculosis* under in vitro conditions. The *Y. pseudotuberculosis* sRNA-ome appears to be distinct from other enteric bacteria and also from the closely related species *Y. pestis*. We have determined that multiple sRNAs are required for the full virulence of *Y. pseudotuberculosis* and that one of these shared RNAs is also required for the full virulence of *Y. pestis*. In addition, we determined that one of the virulence-associated RNAs that is unique to *Y. pseudotuberculosis* controls the abundance of at least eight protein targets. Our study provides new insight into how sRNAs contribute to the pathogenesis of bacteria by regulating the expression of virulence determinants, particularly in pathogenic *Yersinia* species. Additional studies will determine if the gain, loss, or sequence divergence of sRNAs has contributed to the evolution and changing virulence potential of these species.

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References

- Akama T, Suzuki K, Tanigawa K, et al. Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and non-coding regions. *J Bacteriol.* 2009; 191:3321–3327. [PubMed: 19286800]
- Allocati N, Federici L, Masulli M, et al. Glutathione transferases in bacteria. *FEBS J.* 2009; 276:58–75. [PubMed: 19016852]
- Arnvig KB, Young DB. Identification of small RNAs in *Mycobacterium tuberculosis*. *Mol Microbiol.* 2009; 73:397–408. [PubMed: 19555452]
- Busch A, Richter AS, Backofen R. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics.* 2008; 24:2849–2856. [PubMed: 18940824]

- Christiansen JK, Larsen MH, Ingmer H, et al. The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. *J Bacteriol.* 2004; 186:3355–3362. [PubMed: 15150220]
- Coornaert A, Lu A, Mandin P, et al. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol.* 2010; 76:467–479. [PubMed: 20345657]
- Delihans N. Annotation and evolutionary relationships of a small regulatory RNA gene *micF* and its target *ompF* in *Yersinia species*. *BMC Microbiol.* 2003; 3:13. [PubMed: 12834539]
- Fantappie L, Metruccio MM, Seib KL, et al. The RNA chaperone Hfq is involved in stress response and virulence in *Neisseria meningitidis* and is a pleiotropic regulator of protein expression. *Infect Immun.* 2009; 77:1842–1853. [PubMed: 19223479]
- Faucher SP, Friedlander G, Livny J, et al. *Legionella pneumophila* 6S RNA optimizes intracellular multiplication. *Proc Natl Acad Sci USA.* 2010; 107:7533–7538. [PubMed: 20368425]
- Geisinger E, Adhikari RP, Jin R, et al. Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol Microbiol.* 2006; 61:1038–1048. [PubMed: 16879652]
- Geng J, Song Y, Yang L, et al. Involvement of the post-transcriptional regulator Hfq in *Yersinia pestis* virulence. *PLoS One.* 2009; 4:e6213. [PubMed: 19593436]
- Gopel Y, Luttmann D, Heroven AK, et al. Common and divergent features in transcriptional control of the homologous small RNAs GlmY and GlmZ in *Enterobacteriaceae*. *Nucleic Acids Res.* 2011; 39:1294–1309. [PubMed: 20965974]
- Gottesman S, Storz G. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol.* 2010
- Guillier M, Gottesman S. Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol Microbiol.* 2006; 59:231–247. [PubMed: 16359331]
- Heroven AK, Bohme K, Rohde M, et al. A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of *Yersinia pseudotuberculosis* through RovM. *Mol Microbiol.* 2008; 68:1179–1195. [PubMed: 18430141]
- Horler RS, Vanderpool CK. Homologs of the small RNA SgrS are broadly distributed in enteric bacteria but have diverged in size and sequence. *Nucleic Acids Res.* 2009; 37:5465–5476. [PubMed: 19531735]
- Huntzinger E, Boisset S, Saveanu C, et al. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.* 2005; 24:824–835. [PubMed: 15678100]
- Karzai AW, Roche ED, Sauer RT. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat Struct Biol.* 2000; 7:449–455. [PubMed: 10881189]
- Koo JT, Alleyne TM, Schiano CA, et al. Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific sRNAs required for virulence. *Proc Natl Acad Sci USA.* 2011; 108:E709–E717. [PubMed: 21876162]
- Kulesus RR, Diaz-Perez K, Slechts ES, et al. Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli*. *Infect Immun.* 2008; 76:3019–3026. [PubMed: 18458066]
- Kumar R, Shah P, Swiatlo E, et al. Identification of novel non-coding small RNAs from *Streptococcus pneumoniae* TIGR4 using high-resolution genome tiling arrays. *BMC Genomics.* 2010; 11:350. [PubMed: 20525227]
- Landt SG, Abeliuk E, McGrath PT, et al. Small non-coding RNAs in *Caulobacter crescentus*. *Mol Microbiol.* 2008; 68:600–614. [PubMed: 18373523]
- Liu JM, Livny J, Lawrence MS, et al. Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing. *Nucleic Acids Res.* 2009; 37:e46. [PubMed: 19223322]
- Livny J, Waldor MK. Identification of small RNAs in diverse bacterial species. *Curr Opin Microbiol.* 2007; 10:96–101. [PubMed: 17383222]
- Livny J, Brencic A, Lory S, et al. Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2. *Nucleic Acids Res.* 2006; 34:3484–3493. [PubMed: 16870723]
- MacLean D, Jones JD, Studholme DJ. Application of ‘next-generation’ sequencing technologies to microbial genetics. *Nat Rev Microbiol.* 2009; 7:287–296. [PubMed: 19287448]

- Mandin P, Gottesman S. A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. *Mol Microbiol.* 2009; 72:551–565. [PubMed: 19426207]
- Masse E, Vanderpool CK, Gottesman S. Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J Bacteriol.* 2005; 187:6962–6971. [PubMed: 16199566]
- McCullen CA, Benhammou JN, Majdalani N, et al. Mechanism of positive regulation by DsrA and RprA small noncoding RNAs: pairing increases translation and protects *ipoS* mRNA from degradation. *J Bacteriol.* 2010; 192:5559–5571. [PubMed: 20802038]
- Meibom KL, Forslund AL, Kuoppa K, et al. Hfq, a novel pleiotropic regulator of virulence-associated genes in *Francisella tularensis*. *Infect Immun.* 2009; 77:1866–1880. [PubMed: 19223477]
- Moller T, Franch T, Udesen C, et al. Spot 42 RNA mediates discoordinate expression of the *Escherichia coli* galactose operon. *Genes Dev.* 2002; 16:1696–1706. [PubMed: 12101127]
- Morfeldt E, Taylor D, von Gabain A, et al. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *EMBO J.* 1995; 14:4569–4577. [PubMed: 7556100]
- Murphy ER, Payne SM. RyhB, an iron-responsive small RNA molecule, regulates *Shigella dysenteriae* virulence. *Infect Immun.* 2007; 75:3470–3477. [PubMed: 17438026]
- Nakao H, Watanabe H, Nakayama S, et al. *yst* gene expression in *Yersinia enterocolitica* is positively regulated by a chromosomal region that is highly homologous to *Escherichia coli* host factor 1 gene (*hfq*). *Mol Microbiol.* 1995; 18:859–865. [PubMed: 8825090]
- Okan NA, Bliska JB, Karzai AW. A role for the SmpB-SsrA system in *Yersinia pseudotuberculosis* pathogenesis. *PLoS Pathog.* 2006; 2:e6. [PubMed: 16450010]
- Okan NA, Mena P, Benach JL, et al. The *smpB-ssrA* mutant of *Yersinia pestis* functions as a live attenuated vaccine to protect mice against pulmonary plague infection. *Infect Immun.* 2010; 78:1284–1293. [PubMed: 20065026]
- Padalon-Brauch G, Hershberg R, Elgrably-Weiss M, et al. Small RNAs encoded within genetic islands of *Salmonella typhimurium* show host-induced expression and role in virulence. *Nucleic Acids Res.* 2008; 36:1913–1927. [PubMed: 18267966]
- Papenfort K, Pfeiffer V, Mika F, et al. SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol Microbiol.* 2006; 62:1674–1688. [PubMed: 17427289]
- Passalacqua KD, Varadarajan A, Ondov BD, et al. Structure and complexity of a bacterial transcriptome. *J Bacteriol.* 2009; 191:3203–3211. [PubMed: 19304856]
- Pichon C, Felden B. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc Natl Acad Sci USA.* 2005; 102:14249–14254. [PubMed: 16183745]
- Ramirez-Pena E, Trevino J, Liu Z, et al. The group A *Streptococcus* small regulatory RNA FasX enhances streptokinase activity by increasing the stability of the *ska* mRNA transcript. *Mol Microbiol.* 2010; 78:1332–1347. [PubMed: 21143309]
- Rasmussen AA, Johansen J, Nielsen JS, et al. A conserved small RNA promotes silencing of the outer membrane protein YbfM. *Mol Microbiol.* 2009; 72:566–577. [PubMed: 19400782]
- Rowley G, Spector M, Kormanec J, et al. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol.* 2006; 4:383–394. [PubMed: 16715050]
- Schiano CA, Bellows LE, Lathem WW. The small RNA chaperone Hfq is required for the virulence of *Yersinia pseudotuberculosis*. *Infect Immun.* 2010; 78:2034–2044. [PubMed: 20231416]
- Sharma CM, Vogel J. Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol.* 2009; 12:536–546. [PubMed: 19758836]
- Sharma CM, Darfeuille F, Plantinga TH, et al. A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev.* 2007; 21:2804–2817. [PubMed: 17974919]
- Sharma CM, Hoffmann S, Darfeuille F, et al. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature.* 2010; 464:250–255. [PubMed: 20164839]
- Silvaggi JM, Perkins JB, Losick R. Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*. *J Bacteriol.* 2006; 188:532–541. [PubMed: 16385044]

- Sittka A, Lucchini S, Papenfort K, et al. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator Hfq. *PLoS Genet.* 2008; 4:e1000163.
- Sonnleitner E, Sorger-Domenigg T, Madej MJ, et al. Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiology.* 2008; 154:3175–3187. [PubMed: 18832323]
- Srivatsan A, Han Y, Peng J, et al. High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genet.* 2008; 4:e1000139.
- Tjaden B. TargetRNA: a tool for predicting targets of small RNA action in bacteria. *Nucleic Acids Res.* 2008; 36:W109–W113. [PubMed: 18477632]
- Toledo-Arana A, Dussurget O, Nikitas G, et al. The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature.* 2009; 459:950–956. [PubMed: 19448609]
- Tramonti A, De Canio M, De Biase D. GadX/GadW-dependent regulation of the *Escherichia coli* acid fitness island: transcriptional control at the *gadY-gadW* divergent promoters and identification of four novel 42 bp GadX/GadW-specific binding sites. *Mol Microbiol.* 2008; 70:965–982. [PubMed: 18808381]
- Urban JH, Vogel J. Translational control and target recognition by *Escherichia coli* small RNAs in vivo. *Nucleic Acids Res.* 2007; 35:1018–1037. [PubMed: 17264113]
- Urbanowski ML, Stauffer LT, Stauffer GV. The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in *Escherichia coli*. *Mol Microbiol.* 2000; 37:856–868. [PubMed: 10972807]
- Vockenhuber MP, Sharma CM, Statt MG, et al. Deep sequencing-based identification of small noncoding RNAs in *Streptomyces coelicolor*. *RNA Biol.* 2011; 8:3.
- Vogel J, Papenfort K. Small non-coding RNAs and the bacterial outer membrane. *Curr Opin Microbiol.* 2006; 9:605–611. [PubMed: 17055775]
- Vogel J, Sharma CM. How to find small non-coding RNAs in bacteria. *Biol Chem.* 2005; 386:1219–1238. [PubMed: 16336117]
- Wadler CS, Vanderpool CK. Characterization of homologs of the small RNA SgrS reveals diversity in function. *Nucleic Acids Res.* 2009; 37:5477–5485. [PubMed: 19620214]
- Waters LS, Storz G. Regulatory RNAs in bacteria. *Cell.* 2009; 136:615–628. [PubMed: 19239884]
- Yoder-Himes DR, Chain PS, Zhu Y, et al. Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. *Proc Natl Acad Sci USA.* 2009; 106:3976–3981. [PubMed: 19234113]
- Zhang A, Wassarman KM, Rosenow C, et al. Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol.* 2003; 50:1111–1124. [PubMed: 14622403]

Fig. 38.1.

Newly discovered *Yersinia* small RNAs (Ysrs). *Yersinia*-specific sRNAs, comprising 75% of the total, are shown in *dark gray*. Ysrs with orthologs in *E. coli* and *Salmonella*, as determined by BlastN analysis, are shown in *light gray*. *Y. pseudotuberculosis*-specific Ysrs (4%) are represented in *black*. The *right panel* shows that 56% of *Yersinia*-specific sRNAs contain sequence differences between *Y. pseudotuberculosis* and *Y. pestis*

Fig. 38.2.

Contribution of Ysrs to the virulence of *Y. pseudotuberculosis* and *Y. pestis*. **(a)** Groups of 10 mice were orally inoculated with *Y. pseudotuberculosis* wild-type, $\Delta Ysr29$, $\Delta Ysr35$, and $\Delta RybB$ strains ($\sim 2.0 \times 10^5$ CFU). Survival of mice was monitored over 21 days. *P*-values were determined by Mantel–Cox survival analysis log-rank test. *, *P* = 0.0202 for $\Delta Ysr29$ vs. wild type; ***, *P* = 0.0002 for $\Delta Ysr35$ vs. wild type; *P* = 0.9154 for $\Delta RybB$ vs. wild type (not significant). Data are representative of three independent experiments. **(b)** Groups of 10 mice were inoculated via the intranasal route with *Y. pestis* wild type, $\Delta Ysr35$ and $\Delta RybB$ strains ($\sim 1.0 \times 10^4$ CFU). Survival of mice was monitored over 7 days. ***, *P* < 0.0001 for $\Delta Ysr35$ vs. wild type; *P* = 0.0946 for $\Delta RybB$ vs. wild type (not significant). Data are representative of two independent experiments

Fig. 38.3.

Proteomic comparison of *Y. pseudotuberculosis* wild type and Δ Ysr29 strains by 2D-DIGE. The graphic representation of the gel image shows that majority of the spots were within the 1.5 $\times$ differences in spot volume ratio (*vertical lines* flanking the curve). Sixteen marked spots (spots outside the two *vertical lines*) showed differences 1.5 $\times$ or greater between the two strains. Spots to the left of the left 1.5 $\times$ marker represent protein spots with increased expression in the wild-type strain. Spots to the right of the right 1.5 $\times$ marker correspond to the proteins with increased expression in Δ Ysr29 strain. Left Y-axis: number of protein spots; right Y-axis: maximum protein spot volume; X-axis: Cy3 (wild-type)/Cy5 (Δ Ysr29) spot intensity ratio

Table 38.1

Proteins identified in proteomic analysis of Ysr29 mutant and wild-type *Y. pseudotuberculosis* cell lysates by 2D-DIGE/mass spectrometric analysis

Protein	Fold change (mutant vs. wild-type)
DnaK	-7.9
RpsA	-6.2
UreC	1.5
GroEL	-7.0
OmpA	-6.6
AhpC	1.7
GST	2.1
RRF	2.0