Regulation of virulence in *Francisella tularensis* by small non-coding RNAs

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Antisense RNAs regulators in bacteria

Antisense RNAs, ranging in length from 50 to 500 nucleotides, regulate the physiological and virulent functions of bacteria in response to environmental changes and host signals. These small non-protein-coding RNAs (sRNAs) act by basepairing usually modulating the translation (Fig. 1a) and stability (Fig. 1b) of mRNAs.

Figure 1a

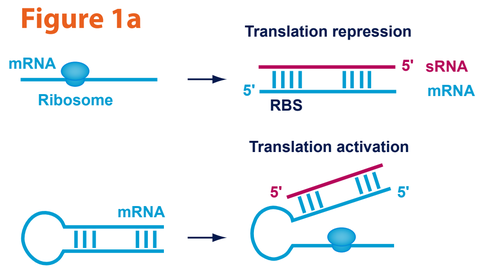
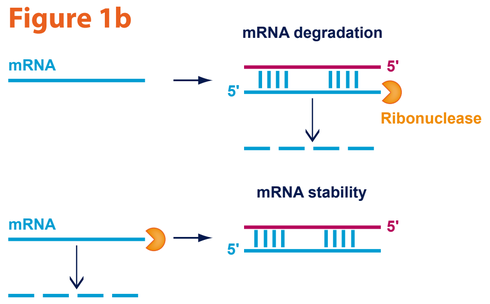


Figure 1b

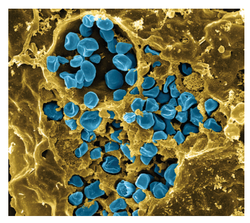


Two classes of bacterial antisense RNAs can be distinguished:
– *cis*-encoded RNAs are located on the strand opposite the target gene, therefore, perfectly complementary to their targets over a long sequence stretch,
– *trans*-encoded RNAs are located in another chromosomal location, with short and only partial complementarity to their target RNAs.

Francisella tularensis, the agent of tularemia

Tularemia is a zoonotic disease caused by *F. tularensis*, a facultative intracellular bacterium, one of the most infectious pathogens known. Less than 10 bacteria are required to cause fulminating disease in both humans and animals.
F. tularensis can initiate infection by most routes, including inhalation, ingestion, dermal micro-abrasions and transmission by insect vectors.
The mortality rate of tularemia may be as high as 30% without antibiotic therapy. Even if not fatal, the disease may be severely incapacitating for a period of weeks or even months.

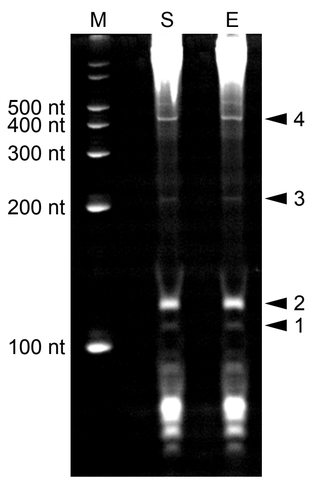
Biological Weapon
Because of its extreme infectivity, ease of aerosol dissemination, and capacity to cause severe illness and death, *F. tularensis* has been classified as a Category A bioterrorism agent.
In the past, *F. tularensis* has been studied, produced, weaponized, and stockpiled by Japan, by the US, and by the Soviet Union.



Relatively little is known about the regulatory networks existing in this organism that allows it to survive in a wide array of environments and **no RNA regulators have been identified** so far.

Francisella tularensis (colored in blue) multiplies within macrophages (adapted from Checroun *et al.*, 2006).

Highly expressed small RNA species



We first assessed the four highly expressed small RNAs visible by PAGE and ethidium bromide staining (Fig. 2; labeled 1–4). The RNA #1 and the RNA #2 were identified respectively as the 4.5S RNA component of the SRP (108 nt) and the 5S ribosomal RNA (114 nt). Despite several attempts, we did not obtain any cDNA clones of RNA #3, the RNA with an apparent size of ~200 nt.

The RNA #4 was identified as the transfer messenger RNA (tmRNA) (Fig. 3). This RNA is also designated SsrA and is 421 nt in size.

Figure 2 | Total RNA extracted at exponential phase (E), stationary phase (S), analyzed by 8% PAGE (M, RNA marker).

Our analysis of highly expressed RNA did not result in the identification of novel small RNA transcripts.

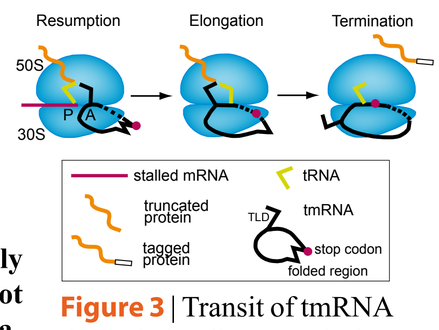
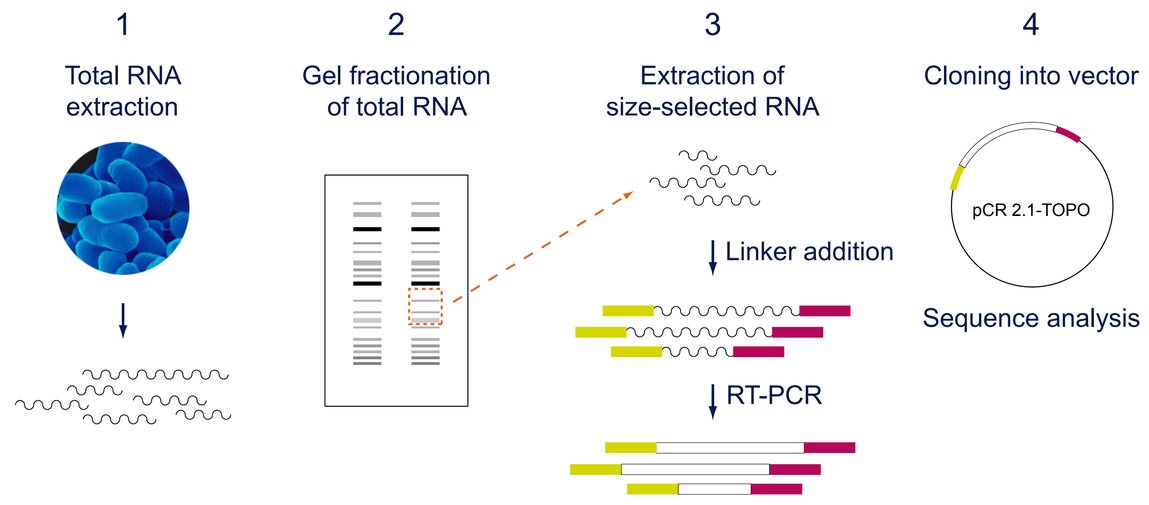


Figure 3 | Transit of tmRNA through the ribosome during *trans*-translation (adapted from Wower *et al.*, 2005).

Shotgun cloning of small-sized RNAs

The identification of novel small RNA species in *F. tularensis* involved the generation of a cDNA library derived from size-selected RNAs.



Identification of two novel small RNAs

By the shotgun cloning of small-sized RNAs (~100 to 200 nt), we identified two different RNAs. We refer to these sRNAs as FtrA and FtrB for *Francisella tularensis* sRNA A and B. **FtrA and FtrB are not similar to any known bacterial small RNAs**.
We performed Northern blotting analysis (Fig. 4), to further confirm these are authentic sRNAs. To map the ends of the RNAs, we performed 5'- and 3'-RACE (Fig. 5). Both FtrA and FtrB are predicted to be highly structured (Fig. 6).

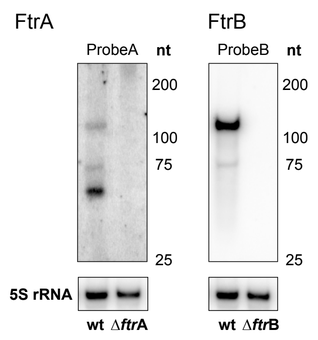


Figure 4 | Northern blots verify the presence of *F. tularensis* sRNAs.

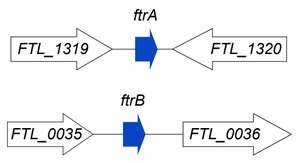


Figure 5 | Schematic representations of the genomic positions of the *ftr*A and *ftr*B loci.

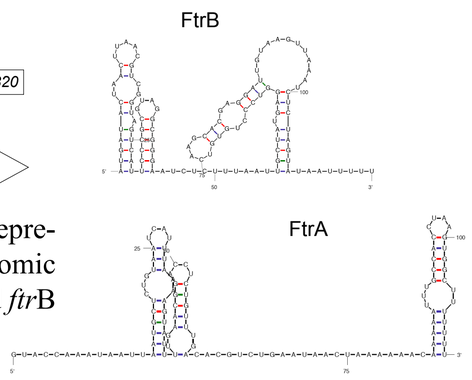


Figure 6 | Predicted secondary structures of FtrA and FtrB.

Characterization of FtrA and FtrB

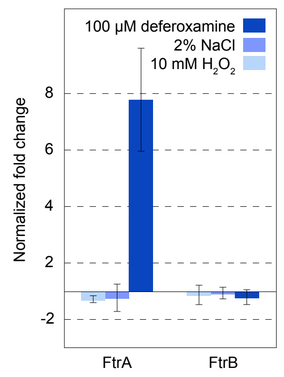


Figure 7 | Effect of several stresses on FtrA and FtrB levels.

Table 1 | Effect of *ftr*A and *ftr*B deletion on the transcriptome of LVS.

To study the role of the sRNAs, we proceeded by creating mutant strains carrying a chromosomal deletion of either the *ftr*A or *ftr*B. Both mutant strains multiplied intracellularly in J774 murine macrophage-like cells in a manner indistinguishable from the wild-type strain. No major difference in survival was observed between mice infected with wild-type LVS or with either of the mutant strains.
Neither mutant strain exhibited any growth defects or increased sensitivity to several stress conditions. However, we observed an increased expression of FtrA in **iron depleted conditions** (Fig. 7).

To experimentally identify potential **targets for regulation** by FtrA and FtrB, we compared the transcriptomes of LVS wild-type bacteria grown in regular broth to that of either the *ftr*A or the *ftr*B mutant (Table 1).

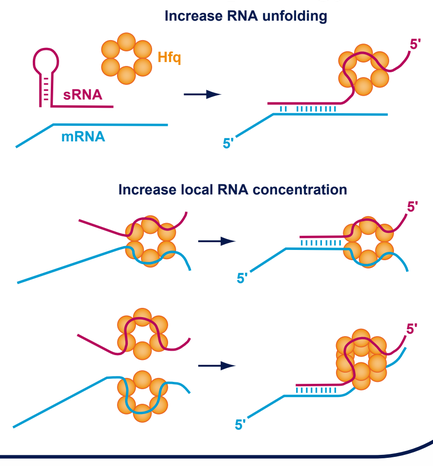
Locus	Gene product	Fold-change in *ftrA*	Fold change in *ftrB*
FTL_0045	orotidine 5'-phosphate decarboxylase	1.8	NA
FTL_0207	pyrrolidone-carboxylate peptidase	2.6	NA
FTL_0902	oxidoreductase	1.8	NA
FTL_1922	YggT family protein	10.8	NA
FTL_0324	pseudogene	NA	0.7
FTL_0421	lipoprotein	NA	0.7
FTL_0836	hypothetical protein	NA	8.2
FTL_1754	hypothetical membrane protein	NA	1.5
FTL_1966	anthranilate synthase component I	NA	0.7

Conclusion and prospects

In this work, we have identified two novel small RNAs, the first in *F. tularensis*. Our results from characterization of the deletion mutants did not enable us to demonstrate that FtrA or FtrB control functions related to virulence. It is important to recall that most sRNAs affect gene expression negatively. Increasing expression of *ftr*A or *ftr*B genes and thus strengthening the repressive effects may have a greater impact on the bacterium.
We have therefore started to generate and characterize two **strains overexpressing *ftr*A and *ftr*B**.

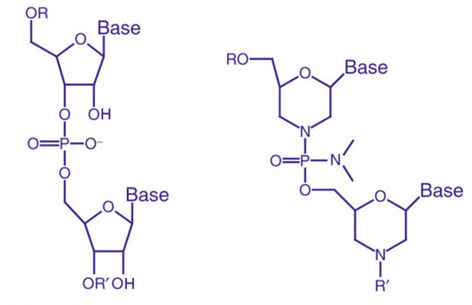
In many bacteria, sRNAs require the bacterial Sm-like **chaperone Hfq** for both intracellular stability and target mRNA pairing (Fig. 8).
To discover new sRNAs, a cDNAs library derived from RNA co-immunoprecipitation with epitope-tagged Hfq will be analyzed, using high-throughput pyrosequencing technology.

Figure 8 | Mechanisms by which Hfq might facilitate sRNA-mRNA basepairing (adapted from Storz *et al.*, 2004).



From natural to artificial systems

Antisense RNA technology has been used in pathogenic bacteria as a **potential therapeutic agent**. Nobel laureate Sidney Altman has developed a strategy using modified oligos (Fig. 9) attached to a cell-permeabilizing peptide (CPP) carrier to inactivate bacterial genes. These RNA-based molecules bind to the bacterial mRNA of choice to activate cleavage through the bacterial enzyme RNase P.



Phosphodiester RNA

Morpholino

Antisense RNA has also been employed in **industrial bacterial systems**, both as a metabolic engineering tool to enhance the productivity of several bacterial hosts and as protection against bacteriophages.

Figure 9 | Chemical structures of phosphodiester RNA and morpholino.

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