Prediction, Microarray and Northern Blot Analyses Identify New Intergenic Small RNAs in *Aliivibrio salmonicida*

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Abstract

Bacterial small RNAs (sRNAs) are *trans*-encoded regulatory RNAs that typically bind mRNAs by short-sequence complementarities and change the expression of the corresponding proteins. Some of the well-characterized sRNAs serve critical steps in the regulation of important cellular processes, such as quorum sensing (Qrr), iron homeostasis (RyhB), oxidative stress (OxyS), and carbon metabolism (Spot 42). However, many sRNAs remain to be identified, and the functional roles of sRNAs are known for only a small fraction. For example, of the hundreds of candidate sRNAs from members of the bacterial family Vibrionaceae, the function is known for only 9. We have in this study significantly contributed to the discovery and verification of new sRNAs in a representative of Vibrionaceae, i.e. the *Aliivibrio salmonicida*, which causes severe disease in farmed Atlantic salmon and other fishes. A computational search for intergenic non-coding (nc) RNAs in the 4.6-Mb genome identified a total of 252 potential ncRNAs (including 233 putative sRNAs). Depending on the set thresh-

old value for fluorescence signal in our microarray approach, we identified 50–80 putative ncRNAs, 12 of which were verified by Northern blot analysis. In total, we identified 9 new sRNAs.

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Introduction

Bacteria contain a diverse set of non-coding (nc) RNAs [Westhof, 2010]. Some of these, such as RNaseP, tRNAs, rRNAs, and tmRNA, serve ‘housekeeping’ functions, CRISPR RNAs protect the bacteria from viruses and plasmids [Horvath and Barrangou, 2010], whereas other classes of ncRNAs serve as regulators in gene expression. *cis*-encoded RNA regulators (i.e. riboswitches) are typically part of mRNAs and located in front of protein coding regions and change the expression of the corresponding protein by binding to small metabolites [Breaker, 2011]. Other regulatory RNAs are transcribed in *trans* from separate promoters located in intergenic regions (IGRs), or from the opposite strand (i.e. anti-sense RNAs) of protein coding regions.

The majority of *trans*-encoded regulatory RNAs in bacteria are small in size (hence the name small RNAs or sRNAs) and act by targeting mRNAs by short imperfect sequence complementarities, thus modulating expression of their targets [Gottesman, 2005]. The result can be change in cellular processes, such as iron homeostasis [Ahmad et al., 2009; Masse and Gottesman, 2002], quorum sensing [Hammer and Bassler, 2007], sugar metabolism [Vanderpool, 2007], oxidative stress [Altuvia et al., 1997], and virulence [Romby et al., 2006]. sRNAs were first discovered in *Escherichia coli* 30 years ago [Griffin, 1971; Ikemura and Dahlberg, 1973], but their abundance and important functional roles have only recently been acknowledged. By the end of the 20th century, only 10 sRNA were known in *E. coli* [Wassarman et al., 1999], but this quickly changed when several research labs started genome-wide systematic searches [Argaman et al., 2001; Chen et al., 2002; Rivas and Eddy, 2001; Wassarman et al., 2001]. After these initial studies in *E. coli*, a number of similar works have resulted in a growing list of sRNAs or putative sRNAs from other bacteria (mostly pathogens), e.g. *Bacillus subtilis* [Irnov et al., 2010], *Vibrio cholerae* [Liu et al., 2009; Livny et al., 2005], *Pseudomonas aeruginosa* [Livny et al., 2006], *Staphylococcus aureus* [Pichon and Felden, 2005], and *Salmonella typhimurium* [Ortega et al., 2012]. As of May 2012, the sRNAMap database lists 79 sRNAs in *E. coli* strain K12 MG1655 (87 in all *E. coli* strains) and a total of 397 in 28 bacterial species [Huang et al., 2009]. Several approaches, such as bioinformatic prediction, microarray, RNA-seq, Hfq coimmunoprecipitation, and RNomics, have proven useful in genome-wide detection of bacterial sRNAs [Sharma and Vogel, 2009], and we expect that a significant number of sRNAs will be discovered in future studies.

The relatively large Vibrionaceae family of gamma-proteobacteria contains a number of serious pathogens of humans (e.g. *V. cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) and animals (e.g., *Vibrio coralliilyticus*, *Vibrio anguillarum*, and *Aliivibrio salmonicida*) [reviewed in Thompson et al., 2004]. These bacteria are therefore of broad interest, and several recent efforts have focused on genome-wide discovery of sRNAs in representatives of Vibrionaceae, mostly using *V. cholerae* as the model [Liu et al., 2009; Livny et al., 2005]. Together, these studies identified hundreds of putative sRNAs and verified the presence of 13 by Northern blot analysis. Experimental evidence of specific roles of sRNA in vibrios and aliivibrios (bacteria from the two genera *Vibrio* and *Aliivibrio*, respectively) is known for Qrr1-5 and the redundant CsrB, CsrC, and CsrD, which all regulate quorum

sensing [Lenz et al., 2005, 2004], RyhB, which is involved in iron homeostasis [Davis et al., 2005], MicX [Davis and Waldor, 2007] and VrrA [Song et al., 2008], which regulates expression of outer membrane proteins, MtlS (previously designated the IGR7 sRNA), which inhibits the synthesis of the mannitol-specific phosphotransferase system [Mustachio et al., 2012], and MRB RNA I, which regulates plasmid replication [Le Roux et al., 2011]. Recently, we presented a study on *A. salmonicida*, in which new roles for Spot 42 sRNA and the discovery of the neighboring VSsrna24 sRNA were presented [Hansen et al., 2012]. Spot 42 appears to have a central role in the central metabolism by regulating the crucial step catalyzed by pyruvate dehydrogenase.

We have in this study performed a genome-wide computational search for ncRNAs in IGRs with special focus on sRNAs in the genome of the Gram-negative and cold-adapted *A. salmonicida*. The bacterium is the causative agent of cold-water vibriosis (or Hitra disease) in farmed Atlantic salmon, sea farmed rainbow trout, and captive Atlantic cod. Computational predictions were used for custom design of a microarray chip containing ncRNA predictions, as well as nearly all open reading frames. Twenty-eight ncRNAs, including 26 previously unrecognized sRNAs, were tested by Northern blot analysis.

Materials and Methods

Bacterial Strains and Growth Conditions

A. salmonicida LFI1238 [Hjerde et al., 2008] and an isogenic $\Delta litR$ mutant [Bjelland et al., 2012] were cultured in LB medium containing 2.5% NaCl at 12°C and 200 rpm. In three independent experiments (biological replicates), cells were subjected to H₂O₂ or 2,2'-dipyridyl after growth in 200-ml culture flasks to mid-exponential phase (OD_{600 nm} ~0.5). Cells were next split into three equally sized cultures. H₂O₂ was added to one flask to a final concentration of 100 µM, the iron chelator 2,2'-dipyridyl was added to one flask to a final concentration of 50 µM, and, finally, one flask was used as the non-treated control. Two 5-ml samples were collected 7.5, 15, and 30 min after treatment, and cells were harvested by centrifugation (3,500 g, 10 min), flash frozen, and kept at -70°C prior to RNA isolation. The *A. salmonicida* $\Delta litR$ mutant was used to find sRNAs with potential roles in quorum sensing. In three independent experiments (biological replicates), the $\Delta litR$ mutant was grown in 60-ml cultures and samples were collected at OD_{600 nm} 0.15, 0.5, and 0.8 for microarray analysis, and OD_{600 nm} 0.15, 0.5, 0.8, and 1.2 for Northern blotting. Wild-type strain was used as control. Samples were subsequently treated as described above.

Northern Blot Analyses

Total RNA was isolated from bacterial cultures using isolation reagent (5 PRIME) and quantified with NanoDrop (Thermo Fisher Scientific). Approximately 10 µg of total RNA was sepa-

rated on 5% polyacrylamide/8 M urea denaturing gels and transferred to a positively charged Hybond-N+ nylon membrane (GE Healthcare) by a TE70 series SemiPhor Semi-Dry Transfer Unit (Hoefer Inc.). Subsequent procedures were done essentially as previously described [Ahmad et al., 2009]. Briefly, RNAs were detected on membranes by using [α - 32 P] dCTP-labeled double-stranded DNA probes, and signals were collected on phosphorimaging screens (Fujifilm) and scanned on a BAS-5000 phosphorimager (Fujifilm). The ImageGauge software v4.0 (Fujifilm) was used to measure the strength of signals. 5S ribosomal RNA was used to normalize the resulting values.

Microarray Analyses

Microarray analysis was run as described in Pedersen et al. [2010]. Briefly, RNA preparations from three independent experiments (biological replicates) were subjected to DNase treatment (to remove DNA) and a clean-up step using RNeasy MinElute spin columns (Qiagen). The three independent RNA samples were then pooled before cDNA synthesis. The quality of RNA was tested, and cDNA was constructed from 15 μ g purified RNA using the Aminoallyl cDNA Labeling Kit (Ambion) and labeled using CyDye™ Post-Labeling Reactive Dye Pack (GE Healthcare). Labeled samples were hybridized to three '*Vibrio salmonicida* V1.0.1 AROS' slides (Eurofins; technical replicates), and slides were subsequently washed. Slides were finally scanned, and resulting expression data were analyzed using J-Express Pro v2.7 [Dysvik and Jonassen, 2001]. Microarray data have been uploaded to The NCBI Gene Expression Omnibus database and are available through accession number GSE25558. Expression data from H₂O₂-treated cells were extracted from GSE20082 [Pedersen et al., 2010].

Computational Prediction of sRNAs

The search for potential sRNA genes in *A. salmonicida* was limited to IGRs. We define an IGR as the region between two annotated genes on either strand that is delimited by its closest left and right flanking genes. Briefly, our sRNA genes were identified by searching for co-localization of genetic features that are associated with bacterial sRNA genes. These include (1) the presence of putative promoters and/or transcription factor binding sites 50–500 nt upstream of Rho-independent terminators, (2) sequence conservation between phylogenetically related species, and (3) conservation of synteny of flanking genes.

BProm (Softberry, Mount Kisco, N.Y., USA; <http://www.softberry.com>), a bacterial σ^{70} promoter recognition program, was used to predict -10 and -35 promoter sequences. Specific position-specific scoring matrices for σ^{54} and σ^{38} were used as input to Patser [Hertz and Stormo, 1999] to scan the *A. salmonicida* IGRs for σ^{54} and σ^{38} promoter sequences. Prediction of potential Fur-binding sites was done as previously described [Ahmad et al., 2009]. TransTerm [Ermolaeva et al., 2000] was used to predict Rho-independent terminators in both chromosomes of *A. salmonicida*. TransTerm searches were conducted with the confidence threshold of 90%. The genome viewer and annotation tool Artemis [Rutherford et al., 2000] and a python script was used to visualize the predicted sequence features described above. The ncRNA prediction programs sRNAfinder [Tjaden, 2008] and QRNA [Rivas and Eddy, 2001] were run for comparison and validation of our predictions.

Compilation of a Vibrionaceae IGR Database and Search for sRNA Homologs

The IGRs from *A. salmonicida* were used to search for homologous IGRs in the six Vibrionaceae genomes of *V. cholerae* O1 biovar eltor str. N16961, *V. fischeri* ES114, *V. parahaemolyticus* RIMD 2210633, *V. vulnificus* CMCP6, *V. vulnificus* YJ016, and *Photobacterium profundum* SS9. Sequences were extracted from GenBank (<ftp://ftp.ncbi.nlm.nih.gov/genbank/genomes/Bacteria/>). An IGR database was constructed based on these six genomes and gene annotation as provided at NCBI's Complete Microbial Genomes (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>). Only IGRs ≥ 50 nt in length were used. The Formatdb option was used to format the IGR database. StandAlone BLAST from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/blast/>) was used to search for homologs of putative sRNAs. Blastn search parameters were set to word size = 7 and E-value ≤ 0.1 .

The search for homologs of known ncRNAs was performed using the cmsearch program, which is part of the Infernal software package [Nawrocki et al., 2009] and the Rfam database of ncRNA families [Gardner et al., 2009]. The lower cmsearch cutoff score was set to 25. Sequences corresponding to tRNAs and rRNAs were excluded.

Results and Discussion

A. salmonicida Total RNA Is Rich in RNAs of 150–200 nt

To evaluate the potential for finding new sRNAs in *A. salmonicida*, we initially cultivated the bacterium and sampled cells at early, mid-, and late exponential phase (i.e. OD_{600 nm} 0.15, 0.75, and 1.0). Total RNA from these samples was separated on a 5% polyacrylamide/8 M urea gel (fig. 1). Major bands were identified as 23S, 16S, and 5S rRNAs and multiple tRNAs based on predicted sizes and previous publications [e.g. Pichon and Felden, 2005]. Interestingly, a number of faint but distinct bands in the size range of 150–200 nt are clearly visible. The size range overlaps well with the expected sizes of bacterial sRNAs [Huang et al., 2009]. To evaluate if at least a fraction of these bands could correspond to sRNAs or other types of ncRNAs, we isolated RNAs of 150–300 nt in length from the gel, and cloned the corresponding cDNA in a shotgun approach. Sanger sequencing of 110 clones revealed that the majority of these corresponded to rRNA (60%) or mRNA (25%) fragments, and only five clones mapped to IGRs. In summary, our initial survey showed that *A. salmonicida* total RNA contains numerous sRNA species. However, the majority of these correspond to rRNA or mRNA fragments, and only a very limited number (~4%) map to IGSs (i.e. potential sRNAs).

Bioinformatic Prediction of 252 Putative ncRNAs

The experimental data described above provided us with limited information about the potential for finding sRNAs, so in a parallel approach, we used bioinformatics to predict ncRNA genes in IGRs. The *A. salmonicida* genome consists of two chromosomes and four plasmids [Hjerde et al., 2008], and we extracted a total of 3,308 IGRs constituting 23% of the genome. We considered only IGRs ≥ 50 nt in length, which delimited the number of IGRs to 2,488 (approx. 21% of total genome). The majority of these (1,828) map to chromosome I.

A search in Rfam identified 25 homologs of known ncRNAs (online suppl. table S1 with detailed information on predicted ncRNAs from intergenic regions of chromosomes I and II in *A. salmonicida*; for all online suppl. material, see www.karger.com/doi/10.1159/000345769) distributed among the categories sRNAs, riboswitches, RNaseP, and tmRNA (excluding tRNAs and rRNAs). Next, putative sRNAs were predicted based on three main criteria: (1) colocalization of putative promoters and/or transcription factor binding sites and Rho-independent terminators (50–500 nt apart), (2) high sequence conservation between relatively closely related species, and (3) conservation of synteny of flanking genes. Figure 2 shows how the 252 predicted ncRNAs (196 in ChrI and 56 in ChrII) are distributed in size, ranging from 65 to 392 nt. The majority is between 101–150 nt and 151–200 nt (73 and 92, respectively), and 87% are ≤ 250 nt, which is in agreement with the typical size of sRNAs (233 of 252 predicted ncRNAs are candidate sRNAs). Promoters were predicted upstream of 48 by BProm and Patser; Fur-boxes were predicted upstream of 14, and 89 were found associated with Rho-independent terminators using TransTerm. Compared to other available bioinformatic sRNA prediction tools, 192 of the predicted sRNAs were recovered by either sRNAfinder or QRNA. Finally, 222 are also found in one or more members of the Vibrionaceae family.

Global Validation of ncRNA Predictions Using Microarray

Next, we wanted to validate our ncRNA predictions on a global scale and used a custom-designed microarray (*Vibrio salmonicida* V1.0.1 AROS) from Operon (now Eurofins) based on 70-mer oligos. The microarray contains oligos complementary to all the 252 predicted ncRNAs (and nearly all the 4,282 protein coding genes, which are not considered here). We assumed that only a fraction of sRNAs would be expressed under normal lab conditions, and we therefore subjected *A. salmonicida* to

Fig. 1. *A. salmonicida* total RNA from early, mid- and, late exponential growth phase separated on a 5% denaturing polyacrylamide gel and stained with ethidium bromide. The positioning of 23S, 16S, and 5S rRNAs and tRNAs was inferred from the predicted sizes. A number of distinct bands can be seen in the size range of approximately 150–200 nt. The asterisk indicates a band of approximately 300 nt that corresponds to a 23S rRNA fragment that was identified by gel isolation, cDNA synthesis, and cloning.

low iron conditions and oxidative stress. Cells were grown to mid-exponential phase ($OD_{600\text{ nm}} \sim 0.5$), subjected to sub-lethal stress conditions by adding 50 μM 2,2'-dipyridyl and 100 μM fresh hydrogen peroxide, respectively, and samples were collected 15, 30, and 60 min after the addition of chemicals. Treated samples were compared with untreated control samples. To find sRNAs with potential roles in quorum sensing, we also analyzed gene expression in a LitR deletion mutant (ΔlitR) of *A. salmonicida* [Bjelland et al., 2012] (the ΔlitR mutant will be described in detail elsewhere). LitR is the homolog of HapR from *V. cholerae*, which is the master regulator of quorum sensing [reviewed in Milton, 2006]. Here, samples were collected at low, mid, and high exponential growth phase ($OD_{600\text{ nm}}$ 0.15, 0.5, and 0.8, respectively) and compared with identical samples of wild type.

Table 1 shows a summary of the microarray results (complete microarray datasets with fold change and fluo-

Fig. 2. Size distribution of predicted ncRNAs. Predicted ncRNAs are shown in dark grey bars, ncRNAs that produced microarray fluorescence signal >2-fold above background are shown in light grey bars, and ncRNAs that produced microarray fluorescence signal >100 are shown in open bars.

Table 1. Summary of ncRNA microarray analysis

	Total ¹	Fold change		Threshold		
		≥2	≥1.5	>50	>75	>100
Low iron	134	7	22	85	68	52
Oxidative stress	142	4	26	91	62	50
<i>ΔlitR</i>	152	15	50	72	60	48

¹ Total number of ncRNAs with fluorescence signal 2× above background.

rescence intensity values for each predicted ncRNA are available in online suppl. table S2), and figure 2 provides a graphical overview of how expressed ncRNAs are distributed into different size categories. Regardless of origin of samples (i.e. treated or untreated samples, or mutant or wild type), 134, 142, and 152 ncRNAs produced fluorescence signals >2-fold above background, and 7, 4, and 15 were differentially expressed under low iron, H₂O₂ conditions, or in *ΔlitR*, respectively. However, these numbers are likely inflated due to unspecific signals and/or false-positive predictions. A more conservative estimate would be to only consider spots with Cy5 or Cy3 fluorescence intensities above certain threshold values. We considered three different threshold values, i.e. >50, >75, and

>100. These were chosen based on the following observations and assumptions: Of the approximately ~4,200 genes that are spotted on the chip, about 3,600 were retained after bioinformatic analysis. With threshold values set at >50, >75, or >100, approximately 1,200, 800, or 700 genes are retained, respectively, which would suggest that at least 33 (1,200/3,600), 22 (800/3,600), or 19% (700/3,600) of genes are expressed under our growth conditions when using microarray technology. These numbers are very modest when compared to recent RNA-seq data from for example *Bacillus anthracis*, which suggests expression of ~94% of the entire genome [Passalacqua et al., 2009]. Even though it is difficult to directly compare data from these two very different technologies, the data indicate that our set threshold value of >100 is relatively conservative, because it is reasonable to believe that more than 19% of genes should be expressed during growth.

Table 1 shows that the average number of spots with fluorescence values above 50, 75, and 100 are 83, 63, and 50, respectively. These spots represent putative sRNAs. Given that the total number of sRNAs in *E. coli* is 87, and that *A. salmonicida* has a similar number of sRNAs, it is reasonable to believe that many of the identified spots could represent genuine sRNAs.

Nine New sRNAs Detected by Northern Blot Analysis

Candidate sRNAs from table 1 were next tested by Northern blot analysis. The experimental setup was iden-

Fig. 3. Northern blot analysis of selected ncRNAs. Radiolabeled double-stranded DNA probes that were specific for predicted sRNAs or one of the three control ncRNAs (Qrr, RNaseP, and 5S rRNA) were hybridized to membranes with total RNA from *A. salmonicida* wild type or $\Delta litR$ mutant. Prior to Northern blot analysis, wild-type cells were grown to mid-exponential phase and treated with 50 μ M 2,2'-dipyridyl or 100 μ M hydrogen peroxide. Untreated (control) cells were used as control. Samples were collected 7.5, 15, and 30 min after treatment. Numbers to the right represent actual and predicted RNA sizes. RNAs from chromosome I (Chr I) or II (Chr II) are indicated. The probe targeted against VSsrna46 produced multiple bands, and band sizes that did not correspond to the predicted size are shown in parentheses. 5S rRNA was used to normalize the strength of signals between lanes as measured with phosphoimaging. The VSsrna185 corresponds to the B2 sRNA that was recently identified in *V. cholerae* [Livny et al., 2005].

tical to that used for microarray, except that cell samples were collected 7.5, 15, and 30 min after treatment (instead of 15, 30, and 60 min) or at ODs 0.15, 0.5, 0.8, and 1.2 (OD 1.2 was added) for the $\Delta litR$ mutant. Probes were designed for 26 putative sRNAs. They were selected because they were highly and/or differentially expressed in the microarray experiment. The Qrr sRNA, RNaseP, and 5S rRNA were included as controls.

Figure 3 shows a summary of resulting Phosphoimage scans of 10 putative sRNAs (VSsrna 7, 21, 108, 145, 152, 185, and 190 on Chr I and VSAsrna 8, 17, and 46 on Chr II) and the three controls. Table 2 shows more details on

these validated RNAs. VSsrna185 is a homolog of the B2 sRNA that was found in *V. cholerae* [Livny et al., 2005]. Predicted and actual RNA sizes are indicated. Thirteen probes did not produce a visible signal, and probes for VSsrna 10, 42, and 115 on Chr I produced multiple bands that could not be resolved and are not shown. As expected, Qrr was differentially expressed in the $\Delta litR$ mutant, from 2- to 5.5-fold upregulation (compared to wild type), which is in agreement with the microarray results (3.4- to 5.6-fold upregulation). In *V. cholerae*, the gene expression levels of Qrr are fine-tuned by two feedback loops that involve HapR (i.e., the LitR homolog) and LuxO,

Table 2. Details of experimentally validated sRNAs

ncRNA ID	Flanking genes ¹	Start ²	End ²	Length ³	Strand ⁴	Sequence conservation ⁵	Promoter ⁶	Terminator ⁷	Type ⁸
VSsrna7	VSAL_I0041/VSAL_I0042	44694	44491	204	<<<	+	–	–	Novel
VSsrna21	VSAL_I0222/VSAL_I0223	267228	267063	166	>>>	+++	SigmaS, Fur-box	+	Novel
VSsrna108	VSAL_I1796/VSAL_I1797	1919211	1919404	194	>>>	+	Fur-box	+	Novel
VSsrna114	VSAL_I1873/VSAL_I1874	2009822	2009717	106	><>	++	Sigma54	+	Qrr
VSsrna145	VSAL_I2441/VSAL_I2442	2615332	2615451	120	>>>	+++	–	–	Novel
VSsrna152	VSAL_I2535/VSAL_I2536	2717523	2717659	137	>>>	++	Fur-box	+	Novel
VSsrna165	VSAL_I2652/VSAL_I2653	2872308	2871972	337	<<<	+++	–	+	RNaseP_bact_a (rnpB)
VSsrna185	VSAL_I2907/VSAL_I2908	3152443	3152724	282	<><	+++	Sigma70	–	B2 <i>V. cholerae</i> , Livny et al. [2005]
VSsrna190	VSAL_I2972/VSAL_I2973	3219049	3219240	192	<><	+++	Sigma70	+	Novel
VSArna8	VSAL_II0231/VSAL_II0232	254998	255222	226	>><	+++	SigmaS	+	Novel
VSArna17	VSAL_II0520/VSAL_II0521	580917	580759	160	<<<	+	–	–	Novel
VSArna46	VSAL_II0920/VSAL_II0921	1006466	1006637	173	>><	+	–	+	Novel

¹ Gene numbers for the up- and downstream *A. salmonicida* open reading frames.

^{2,3} Predicted sRNA coordinates and length (nt).

⁴ Genes encoded on plus strand are denoted with >, and genes encoded on the minus strand are denoted with <.

⁵ Blastn was used to search for sequence conservation in other bacteria. + = Sequence conservation mainly in *Aliivibrio*; ++ = sequence conservation primarily in Vibrionaceae family; +++ = sequence was conserved in many bacterial species.

⁶ Promoter prediction with BProm software and in-house positron-specific scoring matrices and using Patser software [Hertz and Stormo, 1999].

⁷ Rho-independent terminator as predicted by TransTerm software [Ermolaeva et al., 2000].

⁸ sRNAs were queried against Rfam database [Gardner et al., 2009] and other experimentally validated Vibrionaceae sRNAs. Homologs to known sRNAs are indicated.

with HapR indirectly activating expression of Qrr [Svenningsen et al., 2008]. It is unclear to us why the level of Qrr is significantly higher in the *A. salmonicida* LitR mutant, but one possible explanation is that the *litR* mRNA target is absent, and Qrr will therefore not be degraded with its target. It should be noted that the number of *qrr* genes varies between members of Vibrionaceae, for example one in *A. salmonicida* and *Vibrio fischeri*, four in *V. cholerae*, and five in *V. vulnificus* and *Vibrio harveyi* [Miyashiro et al., 2010], which suggests species-specific regulation of *qrr* expression.

Probes targeted to VSsrna7, VSsrna17, VSsrna108, VSsrna152, and VSArna8 each produced one distinct band in close agreement with the predicted size. Probes against VSsrna21, VSsrna145, and VSsrna190 produced, in contrast, bands corresponding to larger RNAs than predicted, which is not unexpected since prediction of promoters and Rho-independent terminators is relatively unreliable, even in the model organism *E. coli*. The VSArna46 probe produced three distinct bands with the strongest band corresponding to an RNA close to the predicted size.

Interestingly, the resulting data suggest that several sRNAs are differentially expressed. In the $\Delta litR$ mutant, VSsrna7, VSsrna108, VSArna8, and VSArna46 are all up-regulated during late exponential phase. VSsrna21 is in

contrast downregulated ~100-fold. The on/off-like expression pattern of VSsrna21 indicates that it might have a direct role in quorum sensing. It does not share significant sequence conservation with any known sRNAs but appears to be conserved in other bacterial species (table 2). Under low iron conditions, VSsrna7 is upregulated 2.4- and 2.9-fold, 7.5 and 15 min after treatment, respectively, whereas VSsrna190 is downregulated after treatment compared to the wild type. Under oxidative stress, VSsrna7 and VSsrna108 are upregulated 2.1- and 2.5-fold, respectively, 15 min after stimulation. The two latter ncRNAs were not noted as differentially expressed using microarray, which demonstrates the importance of using other direct methods, like Northern blot analysis, for verification of results.

In summary, of the 28 microarray spots that were chosen for further investigation using Northern blot analysis, we convincingly identified 11 sRNAs from *A. salmonicida*, including 9 new sRNAs, the homolog of B2 from *V. cholerae*, and Qrr. Six of the new sRNAs are in close agreement with predicted sizes, and 3 are longer than predicted (fig. 3), 8 (VSsrna 7, 108, 145, 152, and 190 and VSArna 8, 17, and 46) produced spots with high intensities (>500) in microarray analysis, whereas one (VSsrna21) produced lower fluorescence signal (i.e. 76). We have tested the majority of putative sRNAs that produced high-intensity spots in microarray analysis using

Northern blot analysis, and we find it reasonable to believe that many of these, if not all, are genuine sRNAs. We expect, however, that many more sRNAs remain to be discovered in *A. salmonicida*, also among the predicted RNAs that were identified with microarray analysis, but that remains to be further tested.

Comparison with Other ncRNA Studies from the Family Vibrionaceae

A few studies have during recent years reported the discovery of ncRNAs in related members of Vibrionaceae [Liu et al., 2009; Livny et al., 2005; Silveira et al., 2010], and we wanted to compare our findings with these results. Twenty-five of our predicted ncRNAs are homologs of known ncRNAs found in Rfam and are distributed among the classes riboswitch (10), RNaseP (1), tmRNA (1), sRNA (6), SRP-RNA (1), anti-sense RNA (1), t44 (1), group II intron (1), and mRNA leaders (3) (see online suppl. table S1 for details). Compared to results from *V. cholera*, our predictions include 14 of 27 previously verified or putative IGR ncRNAs, including 3 of 6 ncRNAs (sRNAPred B2, B4 and C1) that were predicted by sRNAPredict and confirmed by Northern analysis [Livny et al., 2005] and 4 of the 7 IGR sRNAs that were found using direct cloning and parallel sequencing [Sharma and Vogel, 2009]. Of the 7 IGR RNAs from the latter study, our prediction did not include IGR1, IGR4, and IGR6. IGR1 might be present in *A. salmonicida* (Blastn E-value = 0.065 and total score = 32), whereas IGR4 and IGR6 are only found in *V. cholerae*.

Using RNA-seq, Liu et al. [2009] reported 500 IGR RNAs in *V. cholerae* that were considered as putative sRNAs. We removed from this list redundant/overlapping RNA sequences and compared the resulting 251 non-redundant IGR sequences (199 and 52 in chromosomes I and II, respectively) with our 252 predicted ncRNAs using Blastn and the search criteria E-value ≤ 0.01 and total score ≥ 30 . This search identified 21 significant hits (see online suppl. table S1 for details). Nine hits represent homologs of previously known ncRNAs in Rfam (6 riboswitches and 3 sRNAs) and 12 represent novel sRNAs (4 of which have been confirmed with Northern blot analysis).

In a recent study, identification of ncRNAs was performed on environmental vibrios [Rutherford et al., 2000] using a combination of in silico search (Rfam) followed by microarray-based expression profiling. Microarray analysis revealed 21 ncRNAs that were expressed in mid-exponential phase, and a subset of six ncRNAs was tested with reverse transcription PCR. Our predictions identified 17 of these 21 ncRNAs.

Conclusions

We have used computational and experimental methods to identify intergenic ncRNAs (mostly sRNAs) in the marine bacterium *A. salmonicida*, and this is the first genome-wide study of its kind for a fish pathogen. Of the 252 tested ncRNAs, our microarray analysis suggests that approximately ~ 50 (i.e. when using a conservative threshold value) are expressed under our laboratory growth conditions, and Northern blot analysis verified expression of 9 new sRNAs. Our prediction list and expression profiling data also include sRNA candidates recently found in *V. cholerae* with RNA-seq and other methods. One of our current goals is to study the presumably important roles of sRNAs in the development of cold-water vibriosis in Atlantic salmon (and other marine fish species) caused by *A. salmonicida*. By comparing our data with results from other representatives of Vibrionaceae, such as the infamous human pathogen *V. cholerae*, we are discovering sRNAs that are broadly distributed in Vibrionaceae, as well as sRNAs that are limited to aliivibrios and *A. salmonicida* in particular. These analyses will hopefully guide us to better understand the biological roles of sRNAs in this important group of pathogens.

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