A study of regulatory small RNAs in *Vibrio salmonicida*: construction of a knock-out mutant and a cDNA library

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Abstract

The marine fish pathogen *Vibrio salmonicida* is the causative agent for vibriosis in Atlantic salmon (*Salmo salar L.*), Rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua L.*). *V. salmonicidas* virulence is regulated by Quorum sensing (QS) systems, which includes important regulatory small RNAs (sRNAs). sRNAs have the last years been identified in large numbers, and mostly in pathogenic bacteria strains. A cDNA library from small RNA species (120-340 nt) was constructed by size fractionation on a polyacrylamide gel. Due to time limitations, it was not possible to quality check the library properly. More colonies need to be screened in order to decide whether the cDNA library can be used in further studies or not.

One sRNA proven to be very important in QS in several *Vibrios* is the quorum regulating RNA (Qrr). The expression of *qrr* has earlier been confirmed by Northern blotting. Furthermore, *V. salmonicida* has got only one predicted Qrr encoding gene, while other *Vibrios* have several. In order to gain more information about the role of Qrr in *V. salmonicida*, the construction of a *qrr* knock-out by deletion mutant was initiated. The knock-out construct was created, but the conjugation was not successful. The conjugation method for V. salmonicida is not yet established, so further optimization needs to be developed.

A cDNA library of high quality can be subject to further experiments including ultra-high throughtput DNA sequencing followed by expression analysis like Northern blot. A *qrr* knock-out of *V. salmonicida* can be further investigated by methods like Microarray. Discovery of novel sRNAs can give us new insights in *V. salmonicidas* growth, virulence and stress adaptation and reveal possible targets for new antibiotics.

Abbreviations

AI	Autoinducer
AMP	Ampicillin
bp	Base pairs
CAM	Chloramphenicol
CTP	cytosine triphosphate
CIP	Calf intestinal phosphatase
DAP	2,6-Diaminopimelic acid
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
g	g-force
IGR	Intergenic region
LA	Luria agar
LB	Lysogeny broth/ Luria broth
miRNA	microRNA
mRNA	messenger RNA
NaCl	Natruim Chloride
ncRNA	non-protein coding RNA
nt	nucleotides
OD_{600nm}	Optical Density at 600 nanometres
ON	Over night
PA	Polyacrylamide

PAGE	Polyacrylamide gel electrophoresis
PAP	PolyA polymerase
PCR	Polymerase chain reaction
Qrr	Quorum regulatory RNA
QS	Quorum sensing
rRNA	ribosomal RNA
RT	room temperature
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
sRNA	small regulatory RNA
TAP	Tobacco acid pyrophosphatase
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
tRNA	transfer RNA
V	Volt

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Acknowledgement

"I have not failed 1.000 times. I have successfully discovered 1.000 ways to not make a light bulb."
-Thomas Edison-

This quote was an inspiration to keep thinking positive, even though I found 100 ways to not make a transconjugant.

Tromsø, June 1st, 2008
May Liss Julianne Nyrud

1.0   Introduction

1.1   Background: The *Vibrio* genus and *Vibrio Salmonicida*

The genus *Vibrio* constitutes a large and ubiquitous group of aquatic Gamma (γ)-proteobaceria. *Vibrios* are Gram-negative heterotrophic bacteria, shaped as curved rods and typically contain polar flagella. The genus consists of facultatively aerobes that possess a fermentative metabolism (Madigan et al, 2003). Even though *Vibrios* play important roles in nature, for instance in nutrient cycling, they might be best known for their ability to cause disease in humans and marine animals (Thompson et al, 2004). Infections caused by members of the *Vibrio* genus are referred to as vibriosis. Examples of human pathogens in the genus are *V. cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Thompson et al, 2004), *Vibrio anguillarum* (Milton et al, 1997), *Vibrio salmonicida* (Sørum et al, 1990) and *V. vulnificus* (McDougald et al, 2000) are examples of fish pathogens, *V. harveyi* a shrimp pathogen (Liu and Lee 1999) and *Vibrio shiloi* causes bleaching of corals (Rosenberg and Ben-Haim 2002).

Cold-water vibrosis, also called Hitra disease, was first reported in the late 1970s in northern Norway in cultured Atlantic salmon (*Salmo salar L.*) in sea water. The causative agent was later identified as the previously unknown species *V. salmonicida* (Egidius et al, 1986). *V. salmonicida* also causes cold-water vibrosis in rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo Gairdneri*) and Atlantic cod (*Gadus morhua L.*) (Wiik et al, 1989; Sørum et al, 1990). *V. salmonicida* is a marine fish pathogen with an optimal growth temperature of ~15 °C (Colquhoun et al, 2002). It also requires NaCl for growing and can therefore be classified as a psychrophilic and mildly halophilic bacterium.

1.2   Bacterial small regulatory RNAs (sRNAs)

Cellular RNAs can be divided into two main classes according to their function; messenger RNAs (mRNAs), which encode the genetic information necessary for protein synthesis, and non-protein coding RNAs (ncRNAs) (Eddy 1999; Huttenhofer et al, 2002). The latter class accounts for ~98% of the genomic output in humans (Mattick 2001). ncRNAs consists of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) that are both

involved in protein translation, small nuclear RNAs (snRNAs) involved in intron splicing, and small nucleolar RNAs (snoRNAs), which are involved in modification of other RNAs (Huttenhofer et al, 2002). In addition, there are more recently defined classes like microRNAs (miRNAs), small interfering RNAs (siRNAs) and small regulatory RNAs (sRNAs) (Huttenhofer et al, 2002).

Unlike eukaryotic siRNAs and miRNAs, which are defined as a group of antisense regulators sharing a common biogenesis and functional protein components (Filipowicz et al, 2005), the bacterial sRNAs form a heterogeneous class related to their size, structure and function (Vogel and Wagner 2007). sRNAs were first discovered in *Escherichia coli* in 1971 and named '4.5 S', but their function was not yet known (Griffin et al, 1971). The last few years this group has been identified in unexpectedly large numbers. sRNAs are involved in various biological processes, including RNA processing, mRNA protein stabilisation and protein secretion (Huttenhofer and Vogel 2006). sRNAs function by either binding to a protein to modify its activity or act as anti sense regulators by basepairing with mRNAs (Gottesman et al, 2006; Majdalani et al, 2005). sRNA basepairing with mRNA will either result in activation or inhibition of translation, and/or activation or inhibition of mRNA degradation (Majdalani et al, 2005). sRNAs that degrade mRNAs are most studied and almost always interact with the RNA chaperone Hfq (Gottesman et al, 2006). Up until 2001, only 10 sRNA genes were suggested in the model organism *E. coli* (Wassarman et al, 1999). There are now more than 80 sRNAs discovered in *E. coli*, and almost 140 in total in bacteria (Altuvia 2007). It exist different approaches for identifying ncRNAs. The majority of sRNA genes have been discovered by systematic genome analysis based on; i) computational predictions, ii) microarray experiments, iii) shotgun cloning of cDNA libraries, iv) classical cloning of abundant small RNAs after size fractionation in polyacrylamide gels and v) copurification with proteins like Hfq, CsrA and RNA polymerase (Vogel and Sharma 2005; Huttenhofer and Vogl 2006). sRNAs are often found in intergenic regions (IGR) of the genome, and many sRNAs are encoded in the opposite direction of the two encoded flanking genes; like *oxyS* in *E. coli* (Vogel and Sharma 2005; Hersberg et al, 2003). Furthermore are these intergenic sRNAs transcribed from their own promoters and transcription is often

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terminated by a Rho-independent terminator (Figure 1; Vogel and Sharma 2005). This information is especially useful when it comes to computational predictions.
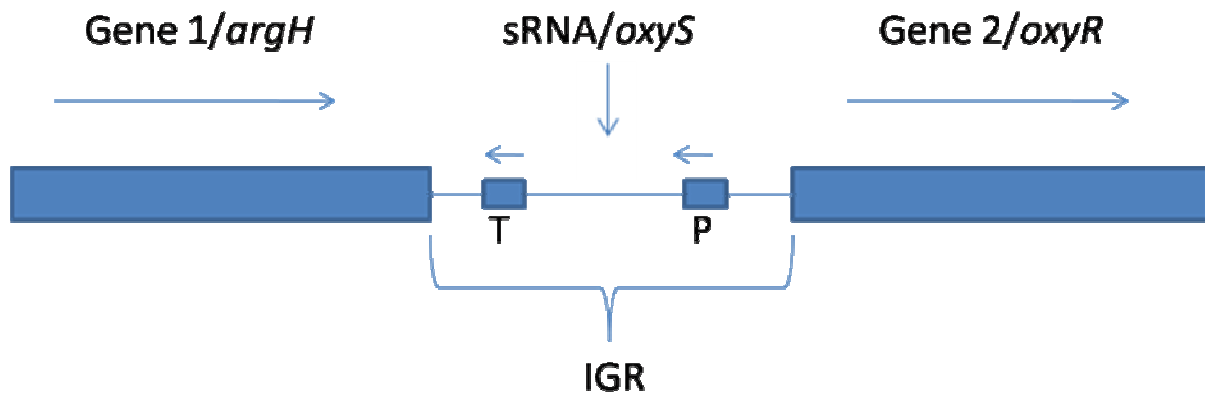
Figure 1: Simplified illustration of an sRNA encoded in an intergenic region (IGR). The sRNA in the IGR is transcribed from its own Promoter (P), and transcription is terminated by a Rho-independent terminator (T). The illustration shows an example taken from E. coli (Hersberg et al, 2003), where the gene for sRNA OxyS is flanked by the two protein encoding genes *argH* and *oxyR*. This figure applies to many sRNAs, but the direction of the flanking genes varies (Hersberg et al, 2003).

Most sRNAs discovered in *E. coli* origin from pathogenic strains (Romby et al, 2006). In recent genome searches there has also been discovered sRNAs in pathogen *Vibrio*s like *Vibrio cholera* and *Vibrio harveyi* (Lenz et al, 2004). Examples of sRNAs acting as mediators in virulence are the carbon storage regulator B (CsrB) that target the CsrA protein in e.g. *V. cholerae* and Qrr that target *hapR* in *V. cholerae* and *luxR* in *V. harveyi* (Romby et al, 2006).

Variants of CsrB is found in several species including *E. coli* (Majdalani et al, 2005), *V. cholera* (Lenz et al, 2005) and *V. fisheri* (Kulkarni et al, 2006). CsrB in *E. coli* is known to cooperate with CsrC (another sRNA) to inhibit the global regulatory protein CsrA (Majdalani et al, 2005; Romeo 1998). Thus, it is a protein binding sRNA. Unlike many other sRNAs, CsrB does not cooperate with Hfq (Majdalani et al, 2005). CsrB functions like a negative regulator on gluconeogenesis and glycogen metabolism (Majdalani et al, 2005). The QS regulating CsrA/B/C signalling system has been suggested in *V. salmonicida* (Hjerde et al, unpublished manuscript).

1.3   Quorum sensing

Quorum sensing (QS) is a process of cell- cell communication that enables bacterial populations to collectively control gene expression and thus coordinates group behaviours (Waters and Bassler 2005). QS is achieved by the synthesis, secretion and detection of signal molecules called autoinducers (AI) that act in parallel and accumulate in proportion to increasing cell density (Hammer and Bassler 2007). *Vibrios* that communicate via QS systems known so far are *V. fischeri*, *V. harveyi*, *V. cholerae*, *V. anguillarum* and *V. vulnificus* (Figure 2; Thompson et al, 2004). The genome of *V. salmonicida* also contains the *lux* operon with homologues of QS regulatory genes (Nelson et al, 2007; Hjerde et al, unpublished manuscript).
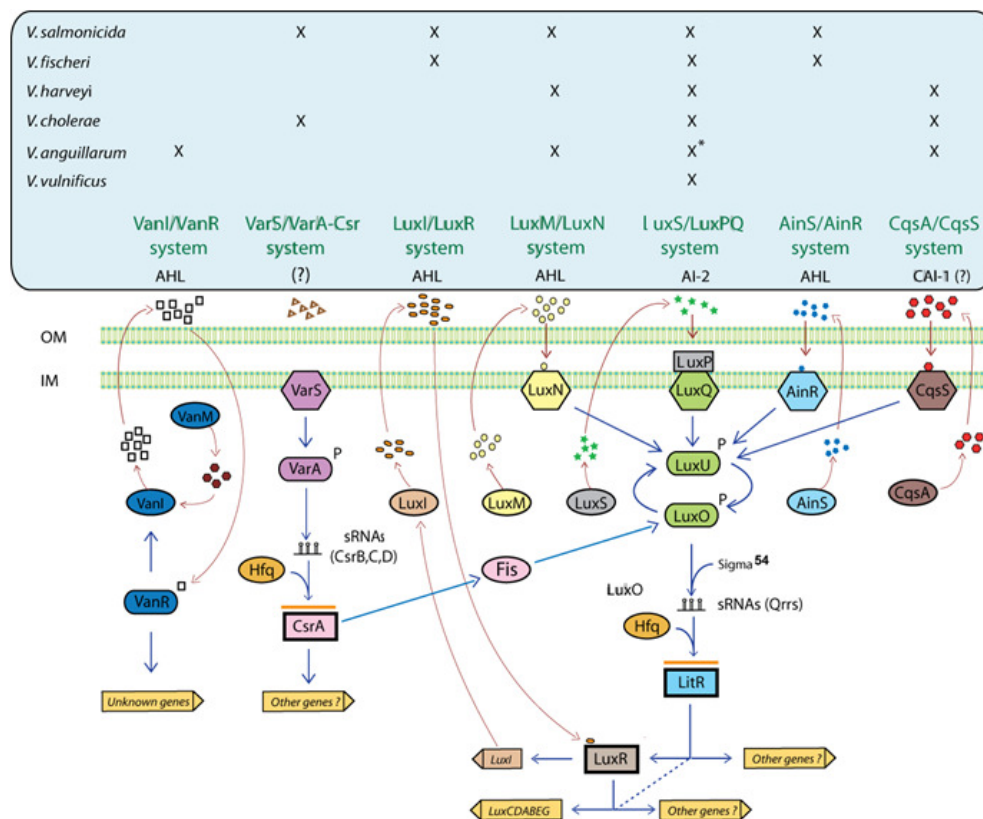
**Figure 2: Schematic overview of the known QS systems in *Vibrios*.** Systems utilized in *V. harveyi* are described in detail in the below text because of the similarity to *V. salmonicidas* predicted QS systems. The LuxM/LuxN system is in the below text referred to as HAI-1. The figure is kindly provided by Prof. Nils-Peder Willassen. *V. anguillarum utilises a system homologous to the LuxS-LuxPQ system.

There are no published results confirming QS systems in *V. salmonicida*, thus *V. Harveyi* and *V. cholerae* are used as examples because they are proposed to be homologous with that of *V. salmonicida*. Some of the processes regulated by QS in the marine bacterium *V. harveyi* are metalloprotease (Mok et al, 2003), type III secretion (Henke and Bassler 2004a), exopolysaccharide production (Lilley and Bassler 2000) and bioluminescence (Bassler et al, 1993; Bassler et al, 1994a; Henke and Bassler 2004b). *V. harveyi* uses three different AIs to control gene expression; HAI-1, CAI-1 and AI-2 (Tu and Bassler 2007). These three AIs are each detected by their own membrane protein; HAI-1 is detected by LuxN, CAI-1 is detected by CqsS and LuxQ responds to AI-2 via the periplasmic protein LuxP (Bassler et al, 1993; Bassler et al, 1994a; Freeman et al, 2000; Miller et al, 2002; Henke and Bassler 2004b). All three membrane proteins phosphorylise a protein called LuxU, which again transfer the phosphor group to the σ^{54}- dependent response regulator protein LuxO (Bassler et al, 1994b; Freeman and Bassler 1999a, 1999b; Lilley and Bassler 2000). LuxO functions by indirectly regulating the expression of *luxR* (*litR* is the homologue in *V. salmonicida*), which again directly activates the *lux* operon (Showalter et al, 1990). Expression of the *luxR* gene causes a 350-fold increase in luciferace mRNA levels (Swartzman et al, 1992). When AIs are in low concentration, the three membrane proteins (LuxN, CqsS and LuxQ) act as kinases and phosphorylise LuxU, which again transfer phosphate to LuxO (Bassler et al, 1994b; Freeman and Bassler 1999a; Lilley and Bassler 2000). The phosphatised LuxO, associated with σ^{54}, indirectly represses the expression of genes encoding luciferase (*lux*) (Freeman and Bassler 1999a; Lilley and Bassler 2000). Therefore no lights are produced. At high cell density, i.e. high AI concentration, the three sensors (LuxN, CqsS and LuxQ) act as phosphatases, leading to dephosphorylation of LuxO. This inactivates LuxO, and leads to expression of the *lux* operon and bioluminescence (Figure 3; Bassler et al, 1994b; Freeman and Bassler 1999a, 1999b; Lilley and Bassler 2000).

As mentioned above, LuxO indirectly regulates the expression of l*uxR*. When LuxO is phosphorylised, it activates the expression of genes called small regulatory RNAs called Qrr (quorum regulatory RNA). Only one Qrr is predicted in *V. salmonicida* (Hjerde et al, unpublished manuscript). Five Qrr sRNAs have been identified and characterized in *V. harveyi* (Tu and Bassler 2007). Four out of these five Qrrs are required to destabilize the

luxR mRNA, and the presence of each individual sRNA causes V. harveyi to express a distinct level of density dependent bioluminescence. This suggests that each Qrr alone is capable of repressing *luxR* to a particular degree (Tu and Bassler 2007). sRNAs like Spot 42 and RyhB are known to interact with the chaperone protein Hfq, to be able to target their mRNAs (Møller et al, 2002; Masse and Gottesman 2002). This is also proposed about the four Qrrs in V. cholera (Lenz et al, 2004), and now about Qrrs in V. harveyi (Tu and Bassler 2007).
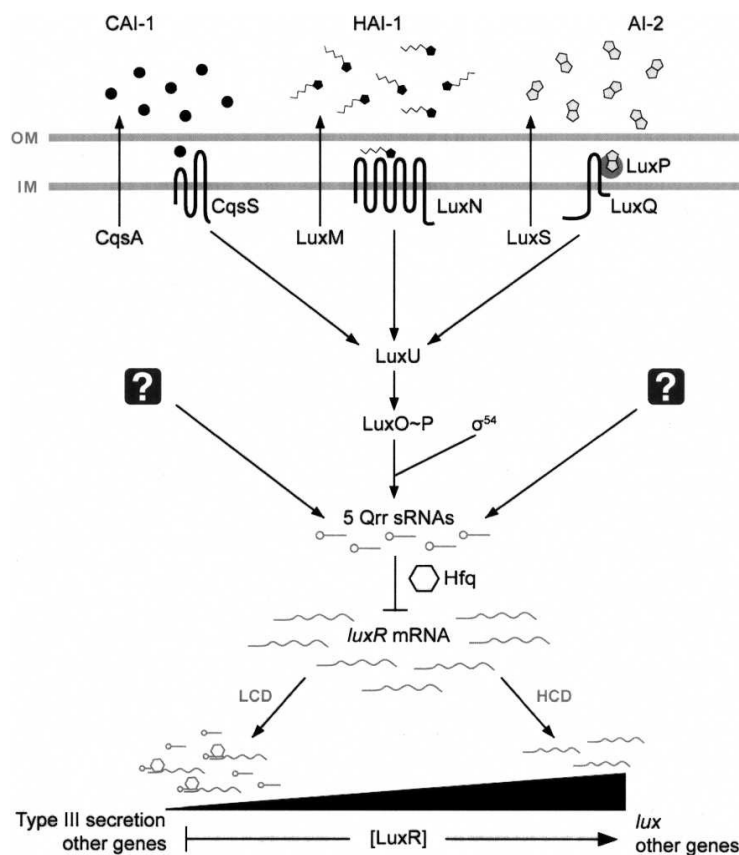
Figure 3: Proposed model for V. *harveyi*s quorum-sensing system. *luxR* controls all known quorum-sensing target genes. *luxR* is regulated by three signalling pathways, each activated by a unique auto-inducer (AI). See text for details. The three AIs are CAI-1 (circles), HAI-1 (pentagons with side chains) and AI-2 (double pentagons). The Qrr sRNAs (lollypops), together with Hfq (hexagons), indirectly regulate the LuxR protein by destabilizing *luxR* mRNA. At low cell density, expression of genes coding for Qrr is activated and LuxR mRNA is destabilized. At high cell density, the opposite happens and genes for quorum-sensing are turned on. Question marks indicate unknown regulators that control expression of Qrr. Figure adapted from Tu and Bassler, 2007.

1.4 Aim of Study

Today it is evident that small non-translated bacterial RNAs are essential for turning on and off virulence genes in pathogenic bacteria, for example by stimulating expression of superoxide dismutases in response to the release of oxidative radicals from host leucocytes. This study consists of two main goals.

Partial goal 1: To get a better understanding of the molecular mechanisms in *V. salmonicida* virulence we wish to identify novel sRNAs, and determine their biological roles. Generating a cDNA library from small RNA species will serve as a step on the ladder of identifying novel sRNAs.

Partial goal 2: To construct a *qrr* knock-out by deletion mutant.

2.0   **Material and Methods**

2.1   **Bacterial strains, plasmids and growth media.**

V. salmonicida strain LFI1238 (originally isolated from Atlantic cod) was utilized in this study. The competent *E. coli* strains DH5α (Invitrogen) and JM109 (Promega) were used for transformation purposes. Also competent *E. coli* strains S17-1 and β-2155, used for *qrr* knock-out construction were kindly provided by Prof. Debra Milton, University of Umeå, Sweden. Both these strains carry the *pir* gene, which is needed for replication of the suicide vector pDM4 (Milton et al, 1995) and strain β-2155 is in addition a 2,6-Diaminopimelic acid (DAP) auxotroph. DAP is essential for cell wall synthesis, and must therefore be used in medium when growing DAP auxotroph *E. coli* strain β-2155.

Vector picric-TOPO (Invitrogen) and poem-T Easy Vector (Promega) were used for transformation into *E. coli* strains DH5α and JM109, respectively. For transformation into *E. coli* strains S17-1 and β-2155, suicide vector pDM4 was kindly provided by Prof. D. Milton, University of Umeå, Sweden. pDM4 contains a chloramphenicol (CAM) resistance gene and requires the *pir* gene for replication and was therefore used in conjugation experiments between *E. coli* strain β-2155 and *V. salmonicida* strain LFI1238. For pDM4 to maintain in *V. salmonicida* after conjugal transfer from *E. coli*, it must be integrated into the chromosome (Milton et al, 1995). This will ensure that only transconjugants grow. The *Vibrio* selective TCBS media is used by Prof. D Milton (1995) to inhibit *E. coli* growth so that only transconjugants of Vibrio can grow. *V. salmonicida* does not grow on this selective media, hence the use of DAP auxotroph *E. coli* β-2155.

In general, *V. salmonicida* was cultured in Lysogeny broth (LB) with 2.5% NaCl at 15°C or grown on blood-agar plates with 2.5% NaCl at 12°C. All *E. coli* strains were grown in LB or on Luria agar (LA) plates with 1% NaCl at 37°C. When growing the DAP auxotroph β-2155, 0.3 mM DAP was added to broth and plates. For broth recipes, see appendix.

2.2   Cultivation of *V. salmonicida*.

From frozen stock, *V. salmonicida* strain LFI1238 was first transferred to blood-agar plates with 2.5% NaCl. These were incubated at 12°C for five days, before colonies were picked and transferred to 20 ml LB with 2.5% NaCl at 15°C, and 220 rpm in a shaking incubator (Multitron, Infors HT). From this initial culture, a similar, but larger 200 ml liquid culture was inoculated at OD_{600nm}=0.10 using a spectrophotometer (Gene Quant pro, GE Healthcare), and grown at 15°C. Growth was then monitored by OD_{600nm}.

2.3   RNA isolation by the TRIzol method.

Total RNA was isolated from 5 ml samples of bacterial cultures that were first centrifuged for 20 minutes (min) at 3,500 g (Allegra X-15R centrifuge, Beckman Coulter) in 15 ml Falcon tubes (Nunc). Supernatants were discarded and pellets were immediately frozen at -70°C. To each pellet, 1 ml TRIzol (Invitrogen) was added and the tubes were vortexed until the suspension was homogenized. After an incubation of 5 min in room temperature (RT), 0.2 ml chloroform was added to the suspension and the tubes were shook for 15 seconds (sec). Next, tubes were incubated for 2-3 min at RT and then centrifuged at 12,000 g (approximately 11,000 rpm in a table top centrifuge) for 15 min at 4°C. The upper aqueous phase was transferred into a clean microcentrifuge tube and the RNA was precipitated by adding 0.5 ml isopropanol. The solution was incubated for 10 min at RT and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded, and 1 ml 75% Ethanol (EtOH) was added to the pellet before vortexing. The tubes were centrifuged at 7,500 g (approximately 8000 rpm in a table top centrifuge) for 5 min at 4°C. The supernatant was again discarded, and the pellet was air dried for 5-10 minutes. The pellet was dissolved in 20 μl diethylpyrocarbonate (DEPC)-water and the tubes were incubated for 10 min at 55-60°C. The isolated RNA was stored at -20°C. Diluted RNA was quantified using spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific).

2.4   RNA quality control

To control the quality of isolated total RNA, the Experion RNA StdSens Analysis Kit (Bio-Rad) was used. RNA samples were tested on an Experion RNA StdSens chip and run on an Experion electrophoresis station. The procedure was performed using a local laboratory connected to microarray.

2.5   DNase I treatment.

To remove DNA from isolated RNA, cloned DNase I (Takara Bio Inc) and provided buffer were used. Two parallel reactions were set up both with a total volume of 120 µl containing 300 µg RNA, 75 units DNaseI and 1x DNase buffer. The reaction was carried out at 37°C for 10 minutes. RNA was then precipitated as described below (i.e., section 2.6).

2.6   Phenol-Chloroform extraction and precipitation of RNA

To remove proteins and salt, RNA samples were subjected to Phenol-Chloroform extractions followed by ethanol precipitations. An equal volume of Phenol:Chloroform:Isoamyl Alcohol (P:S:I; 25:24:1) (Invitrogen) was added to RNA samples, which typically had a volume of 100-200 µl. Tubes were then vortexed vigorously and centrifuged for 5 min at full speed on a table top centrifuge at RT. The upper aqueous phase was transferred to a new tube and the RNA was precipitated with 1/10 volume of 3M NaOAc and 2-3 volumes of Absolute EtOH. RNA was then precipitated by placing the tubes at -20°C for a few hours or ON followed by centrifugation for 30 min at full speed on a table top centrifuge at 4°C. The supernatant was discarded and the pellet was washed with 1 ml 70% EtOH. The tube was spun for 2 min at full speed on a table top centrifuge at 4°C, before the remaining EtOH was removed and the pellet was air dried for a few minutes. The pellet was finally dissolved in DEPC-water.

2.7 Polyacrylamide gel electrophoresis (PAGE) for fractionation of RNA

In order to isolate small RNA species (e.g., sRNAs) from total RNA, a protocol based on Aspegren et al, 2004 was followed. A 5% denaturing polyacrylamide (PA) gel (see appendix for recipe) was prepared and mounted on a Sequi-Gen GT Sequencing Cell gel system (Bio-Rad Laboratories). The gel was pre-run for approximately 70 min (i.e., until the gel reached 45°C). The RNA sample was mixed with an equal amount of STOP-solution (see appendix for recipe) and loaded on the gel. RNA Century™ Marker-Plus (Applied Biosystems) was mixed with STOP-solution and used as size marker. Samples were run for 70 min at max 50 Volt (V) and the temperature was set to 45°C. The RNA was next stained with a dilute ethidium bromide (EtBr) solution and visualized by UV-light using a Gel Doc 200 (Bio-Rad Laboratories). RNA was cut from the gel using a ruler and put into a 50 ml Falcon tube (Becton Dickinson). The gel pieces were stored at –20°C.

2.8 Gel elution

RNA was recovered from PA gel-pieces by first adding 6 ml elution buffer (0.1 M NaOAc, 1mM EDTA, 0.5% SDS) to 50 ml Falcon tubes containing RNA/gel. Tubes were then left on a rotating wheel (Stuart SB3, SciLabware/Barloworld Scientific) at 4°C ON. A syringe with a 0.45 µm filter (Pall Corporation) was used to filtrate the solution. The syringe was washed with 500 µl elution buffer both before and after filtration, to ensure that as much RNA as possible was recovered. The sample was split into equal amounts of 500 µl, transferred to 1.5 ml microcentrifuge tubes and precipitated with 50 µl 3M NaOAc, 1 ml 99% EtOH and 10 µg glycogen (Applied Biosystems) at -20°C ON. The samples were then centrifuged for 30 min at full speed on a table top centrifuge at 4°C. The pellets were washed with 1 ml 70% EtOH, briefly centrifuged, air dried and dissolved in 20 µl DEPC-water. The samples were pooled and purified with one round of phenol/chloroform, respectively, and precipitated again as described above (i.e. section 2.6). The sample was measured by a Nanodrop 1000 (Thermo Fisher Scientific), and stored at -20°C.

2.9   rRNA removal

A previous attempt of making an sRNA library from *V. salmonicida* resulted in that the majority (approximately 60%) of clones represented fragments of rRNAs (rRNAs typically represent >80% of total RNA in bacteria). Because of this, rRNA was removed by the MICROB*Express*™ Bacterial mRNA Purification Kit (Ambion) before cDNA library construction. rRNA removal was done according to the manufacturers' protocol.

2.10   cDNA library construction

Full length cDNA library construction was followed by a protocol based on Huttenhofer et al, 2001. To generate a cDNA library from small RNAs, the 3' ends of RNAs were first modified by adding poly(C)-tails using PolyA polymerase (PAP) (GE Healthcare) and cytosine triphosphate (CTP) nucleotides. A reaction contained 5 µg RNA, 0.083 mM $MgCl_2$, 0.083 mM CTP, 1x tailbuffer (0.5 M Tris ph 8.0, 2 M NaCl, 0.1 M $MgCl_2$, 4 mM EDTA, 10 mM DTT, 20 mM $MnCl_2$), 4 U RNase inhibitor (TaKaRa) and 7.5 U PAP in a total volume of 50 µl. Before PAP was added, the regents were prewarmed to 37°C. After the addition of PAP, the reaction was carried out at 37°C for 1 hour.

In order to make cDNA from poly(C)-tailed RNA, GeneRacer™ RACE Ready cDNA Kit (Invitrogen) was used. The library was made according to the manufacturers' recommendations but with modifications as described below. The calf intestinal phosphatase (CIP) treatment was omitted. CIP removes phosphates from the 5' end of truncated mRNAs and non-mRNAs to prevent ligation with an RNA oligo. Because we are interested in non-mRNAs, we did not wish to remove the 5' phosphate. The poly(C)-tailed RNA was treated with tobacco acid pyrophosphatase (TAP), before the provided lyophilized RNA oligo was ligated to the 5' end in the presence of 10% dimethyl sulfoxide (DMSO). DMSO was used to reduce RNA secondary structures. Reverse transcription was performed at 42°C for 30 min and then at 50°C for another 30 min, followed by inactivation of the reaction at 70°C for 15 min. A mixture of oligonucleotides (Oligo dC3'A, Oligo dC3'C and Oligo dC3'T) with a concentration of 16.5 µM each was used for the reverse transcription (see table 1 in appendix for sequences). The primers were customized to anneal to all poly(C)-tailed RNA with repeated C nucleotides at the very 3' end of the RNA. After cDNA synthesis RNA was removed by adding 1 µl of RNase

H (included in Invitrogen kit) and incubating samples at 37°C for 20 min. The cDNA was stored at -20°C.

2.11   Polymerase chain reaction (PCR)

Standard PCR with Taq DNA polymerase (New England BioLabs) was used for colony screenings and primer testing. For colony screening to verify an insert, a colony was dissolved in 10 μl MilliQ (Millipore)-purified water and boiled for 10 minutes at 100°C to generate DNA templates. Samples were then spun for 1 min at full speed on a table top centrifuge, before 1 μl of the supernatant was used as template in the PCR reaction. For other PCR reactions performed on purified plasmids, approximately 10-100 ng DNA was used as template. Added to the template was 0.33 mM dNTP, 0.33 mM forward and reverse primer, 2.5 U Taq DNA Polymerase, Thermo Polymerase Buffer (New England BioLabs) and MilliQ water to a total volume of 30 μl. A standard PCR program was initiated with a 5 minutes incubation of 95°C before 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec were run. A final extension of 72°C for 5 minutes was carried out before the sample was incubated at 4°C indefinite.

2.11.1   PCR performed with proofreading polymerase

If PCR product were used for cloning purposes, a thermostable polymerase with proofreading capabilities was used in a slightly different protocol. Here reactions contained a total volume of 30μl with 1 μl DNA template, 1 U Phusion High-Fidelity DNA Polymerase (New England BioLabs), Phusion HF Buffer (New England BioLabs), 0.33 mM dNTP and 0.33 mM of each primer.

2.11.2   PCR performed to fuse two PCR products

When two PCR products were fused in a PCR, 1 μl was used of each product. The following PCR was used: All reagents except for the primers were mixed, and an initial PCR was set up with a 94°C incubation and 7 cycles of 94°C for 30 sec, 40°C for 45 sec and 68°C for 1 min. Immediately after the initial PCR ended, the two primers were added to the reaction mix and set up on a second PCR with 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 68°C for 1 min. A final extension of 68°C for 5 min was added and the sample was incubated at 4°C indefinite.

To gain 3' A-overhangs required for TA Cloning, the overlap construct was added 1 U Taq polymerase (New England BioLabs) after the PCR. The sample was incubated for 10 min at 72°C, and used immediately in the TOPO TA Cloning reaction as described below (i.e. section 2.18).

2.11.3 Touchdown PCR used for cDNA library amplification

For amplification of the cDNA library, a touchdown PCR was used. Starting with a high annealing temperature followed by lower annealing temperatures through the remaining PCR cycles increase specificity and reduce background amplification. One µl of the first-strand reaction gained in section 2.10 was used as a template. In a total volume of 50 µl the reaction contained 1x Thermo Polymerase Buffer (New England BioLabs), 5 U Taq DNA polymerase, 0.3 mM dNTP mix (Invitrogen) and 0.9 mM of each primer. The touchdown PCR program for cDNA amplification had an initial incubation of 94°C for 2 minutes followed by 5 cycles of 94°C for 15 sec and 72°C for 70 sec; another 5 cycles of 94°C for 15 sec, 69°C for 30 sec and 72°C for 40 sec; and finally 25 cycles of 94°C for 15 sec, 65°C for 30 sec and 72°C for 40 sec. The program was ended with a final elongation at 72°C for 10 min and the samples were incubated at 4°C indefinite.

2.12 Agarose gel electrophoresis and DNA extraction

One percent agarose gels made from 1x Tris-Borate-EDTA (TBE) buffer were used for colony screening and separation of PCR products and plasmids. The gel was added 1.5 µl ethidium bromide (EtBr, 10 µg/ml) and run in 1x TBE buffer for 1 hour at 90V. For DNA extraction from agarose gels, Montage Gel Extraction Kit (Millipore) containing spin columns that separate DNA from agarose gel in a 10 min spin, was used according to manufacturers' protocol. Gels made for the purpose of DNA extraction, were made from and run in a 1x Tris-Acetate-EDTA (TAE) modified buffer, provided with the kit.

2.13   Plasmid purification

Transformed colonies were picked and grown in 1.5 ml LB medium, containing 100µg/ml Ampicillin (AMP). Colonies obtained from the cDNA library were grown in 96-well plates. The overnight cultures were grown on 37°C at 220 rpm (KS 501 digital shaker, IKA Labortechnik). Plasmids from each overnight culture were isolated with the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek), according to the manufacturers' protocol.

2.14   DNA   sequencing

The Big Dye® Terminator version (v) 3.1 Cycle Sequencing Kit (Applied Biosystems) was used for all DNA sequencing approaches. Reactions contained 150-200 ng plasmid DNA template, 0.5 mM M13 Forward Primer (see table 1 in appendix for sequence), 2 µl Big Dye® Terminator v3.1 Ready Reaction Premix, and Sequencing buffer in a total volume of 20 µl. To sequence multiple numbers of different templates the total volume was reduced to 10 µl per reaction, containing same concentration of template and the other reagents. Sequencing reactions were subjected to an initial denaturing step of 1 min at 96°C before cycled 25 times between 96°C for 10 sec, 55°C for 5 sec and 60°C for 4 min. The sample was kept at 4°C indefinite. Reactions were precipitated and run on a 3130 Genetic Analyzer (Applied Biosystems) using a local sequencing facility.

2.15   Bioinformatic approaches

The computer programs Sequence Scanner v1.0 (Applied Biosystems) and BioEdit v7.0.9 (Ibis Biosciences) were used to visualize and perform simple analysis of the cDNA library sequencing data. The genomic viewer and annotation tool Artemis v9 (The Wellcome Trust Sanger Institute; Rutherford et al, 2000) was used to compare cDNA sequence data with the genomic sequence from *V. salmonicida*. Folding of predicted sRNAs was performed by MFOLD (Mathews et al, 1999; Zuker 2003). Obtained sequences of interest were submitted to the sequence databases Basic Local Alignment Search Tool (BLAST) and the RNA families database of alignments and CMs (Rfam) (The Wellcome Trust Sanger Institute; Griffiths-Jones et al, 2005) for further investigation.

2.16   Restriction enzyme digestion

To gain cohesive ends, purified DNA (plasmids or PCR products) were digested with restriction enzymes *Xho*I (cutsite 5'-C^TCGAG- 3') and *Spe*I (cutsite 5'-A^CTAGT- 3') in 20 μl reactions with approximately 60 ng DNA, 1x buffer H (Promega) and 10 U of each restriction enzyme. For digestion of the PCR product, an ON incubation at 37°C was used, whereas the plasmids were incubated for 3 hours.

2.17   Ligation

Ligation of insert into pCRII-TOPO vector (Invitrogen) and pGEM-T Easy Vector (Promega) was done according to manufacturers' protocol. Ligation of insert into suicide vector pDM4 was carried out with a 3:1 insert-vector ratio. The correct ratio according to the products sizes was calculated at http://www.insilico.uni-duesseldorf.de/Lig_Input.html. In a total reaction volume of 10 μl, 3:1 insert-vector, 1x T4 DNA Ligase Reaction Buffer and 400 cohesive ends U T4 DNA Ligase (New England BioLabs) was used. The ligation was carried out at 16°C ON and inactivated at 65°C for 10 min.

2.18   Cloning and transformation of *E. coli* cells

One Shot® DH5α™ competent *E. coli* cells (Invitrogen) and High Efficiency JM109 competent *E. coli* cells (Promega) were used for cloning and transformation purposes according to manufacturers' protocol. Briefly, transformation of competent *E. coli* S17-1 cells was carried out by thawing a tube containing 50μl of cells on ice and adding 1 μl of ligation mix (see section 2.17 above). The cells were incubated for 30 min on ice, heat shocked at 42°C for 45 sec and put on ice for 2 min before 450 μl SOC medium (recipe in appendix) was added to the tube. The cells were incubated at 37°C for 1 hour at 220 rpm (KS 501 digital shaker, IKA Labortechnik), before 50 and 200 μl was plated on LA plates containing 25μg/ml CAM and incubated at 37°C over night. Transformation of the DAP auxotroph *E. coli* β-2155 strain was carried out similar, with the only difference being to add 0.3 mM DAP in both the SOC medium and LA plates.

2.19   Conjugation using the DAP auxotroph *E. coli* β-2155 strain.

Two separate initial ON cultures were grown, one with *V. salmonicida* LFI1238 and the other with DAP auxotroph *E. coli* β-2155 cells transformed with pDM4-qrr. *V. salmonicida* was grown in 20 ml LB with 2.5% NaCl at 12°C and 220 rpm (Multitron, Infors HT). *E coli* was grown in 10 ml LB with 1% NaCl, 25 µg/ml CAM and 0.3 mM DAP at 37°C and 220 rpm (KS 501 digital shaker, IKA Labortechnik). Both bacteria cultures were split and started growing over again at $OD_{600nm}\sim0.1$-0.2. Because *V. salmonicida* is a slow grower, the *E. coli* culture was set up approximately 5 hours after *V. salmonicida*. Both cultures were grown to $OD_{600nm}\sim0.6$, when *V. salmonicida* is in its exponential phase. The cultures were split between microcentrifuge tubes; 7 tubes of 1.5 ml for *V. salmonicida* and 2 tubes of 1.5 ml for *E. coli*. The cells were spun down at RT for 5 min at full speed on a table top centrifuge, and resuspended in LB with 1% NaCl and 0.3 mM DAP. The cells were spun again as above and resuspended in 5 µl LB with 1% NaCl and 0.3 mM DAP, before recipient (*V. salmonicida*) was mixed with donor (*E. coli*) in a 1:1 and 6:1 ratio. The mixed cells were spotted on a LA plate containing 1% NaCl and 0.3 mM DAP, and incubated at RT (approximately 21°C) for 7 hours before moved to 12°C ON (approximately 14 hours). The cells were then resuspended in 1 ml LB with 2.5% NaCl and incubated at 12°C and 220 rpm for 7 hours. Different amounts were plated out on LA plates with 2.5% NaCl and 2 µg/ml CAM. The plates were incubated at 12°C for three-five days before they were examined for small, transparent colonies that could indicate a *V. salmonicida* transconjugant. Potential transconjugants were picked and spread on a LA plate with 2.5% NaCl and 2 µg/ml CAM and again incubated at 12°C for three-five days.

3.0 Results

3.1 Construction of a cDNA library based on small (120-340 nt) RNAs from *V. salmonicida*.

The first main task of this study was to isolate RNA species of about 120-340 nt in size and to construct a cDNA library based on these. By randomly sequencing clones from this library the objective was to identify novel regulatory sRNAs.

3.1.1 *V. salmonicida* growth curve

The cDNA library from *V. salmonicida* was going to be based on cells sampled from late exponential growth phase, because once cells enter the stationary phase RNA can quickly degrade. To gain a perspective of how fast *V. salmonicida* grows and to make an overview of its exponential and stationary phase, a growth curve was established. This would help us decide from which point of the growth phase to isolate RNA. *V. salmonicida* was grown in LB- medium at 15°C, and OD_{600nm} was measured hourly with a spectrophotometer.
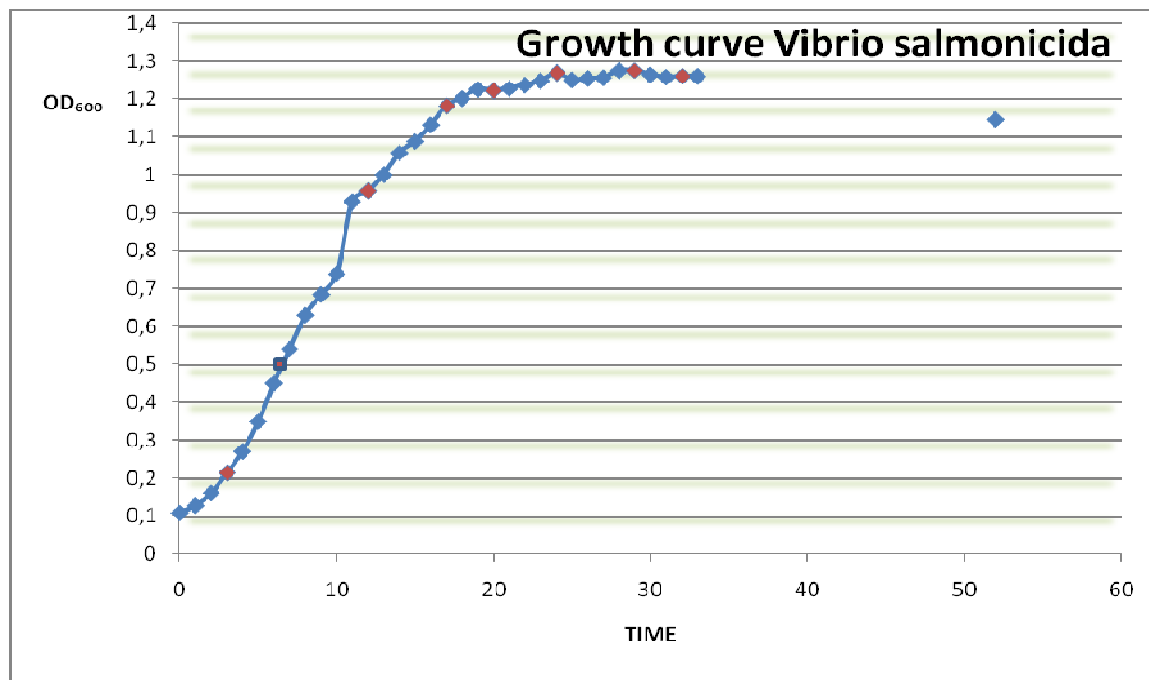

Figure 4: Growth curve for *V. salmonicida*. Samples were taken every hour from a culture of *V. salmonicida* in order to measure the OD_{600nm} and monitor the growth. Red marks indicate samples collected to isolate RNA from..

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The growth curve (figure 4) of *V. salmonicida* shows a mid- exponential phase after approximately 10 hours with $OD_{600nm} \sim 0.75$ and the stationary phase is reached after 24 hours at $OD_{600nm} \sim 1.25$. Five ml bacterial cell cultures were sampled at different OD_{600nm} values to compare amounts of RNA and to see if any distinct RNA bands were detected at particular timepoints.

3.1.2   RNA quality control

In order to decide from which OD_{600nm} value the sRNA library was to be made from, total RNA was isolated from OD_{600nm}= 0.21, 0.51, 0.96, 1.18, 1.22, 1.26, 1.27 and 1.26 (after 32 h), using the Trizol protocol (see section 2.3 for details). To ensure proper RNA quality after isolation, two µl from each of the RNA samples was run on an Experion RNA StdSens chip.
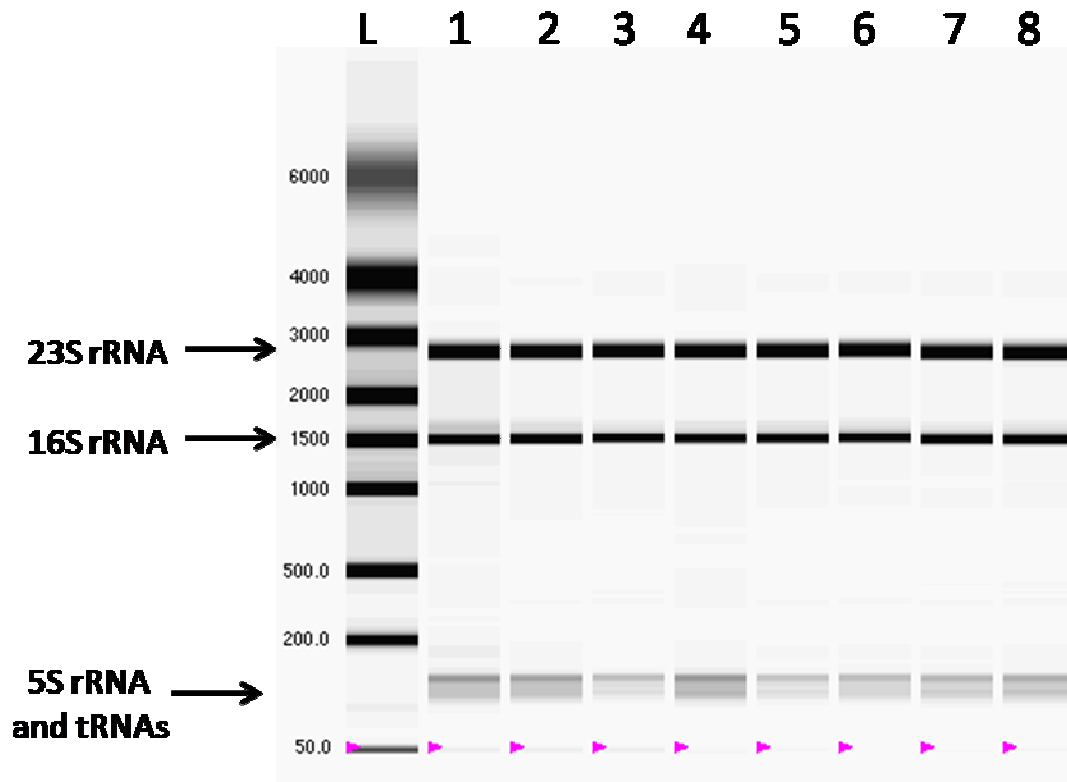
Figure 5: RNA quality control. The quality of the isolated RNA was tested on an Experion RNA StdSens chip. The first lane contains an provided RNA ladder, while lane 1-8 contain samples isolated from OD_{600nm}= 0.21, 0.51, 0.96, 1.18, 1.22, 1.26, 1.27 and 1.26, respectively.

Lanes in figure 5 show as expected distinct peaks of the three rRNA subunits; 5S rRNA (as well as tRNAs), 16S rRNA and 23S rRNA. A low amount of background confirms high purity and low levels of degradation of the RNA. Therefore, this RNA was further tested to decide at which OD_{600nm} the cDNA library should be made. An earlier attempt to create a cDNA library from *V. salmonicida* gave a high percentage of clones representing fragments of rRNAs. This problem was in this study addressed by adding an rRNA removal step. For this purpose, rRNA-specific oligonucleotides attached to magnetic beads were used to remove rRNA fragments. The oligonucleotides were mixed with RNA and left to hybridize for 15 minutes, before the tube was placed on a magnetic rack so that the beads were pulled aside. The supernatant contains mainly mRNA, tRNA, 5S rRNA and other small RNAs. As an initial test, three of the RNA samples were treated using this method. Ten µg RNA was taken out from each sample, both before and after rRNA removal, and compared on a 5% denaturing PA gel (figure 6). If RNA originating from a particular OD_{600nm} value would show a unique band pattern, or distinct RNAs that were highly expressed, then it could be interesting to make an sRNA library from such samples.
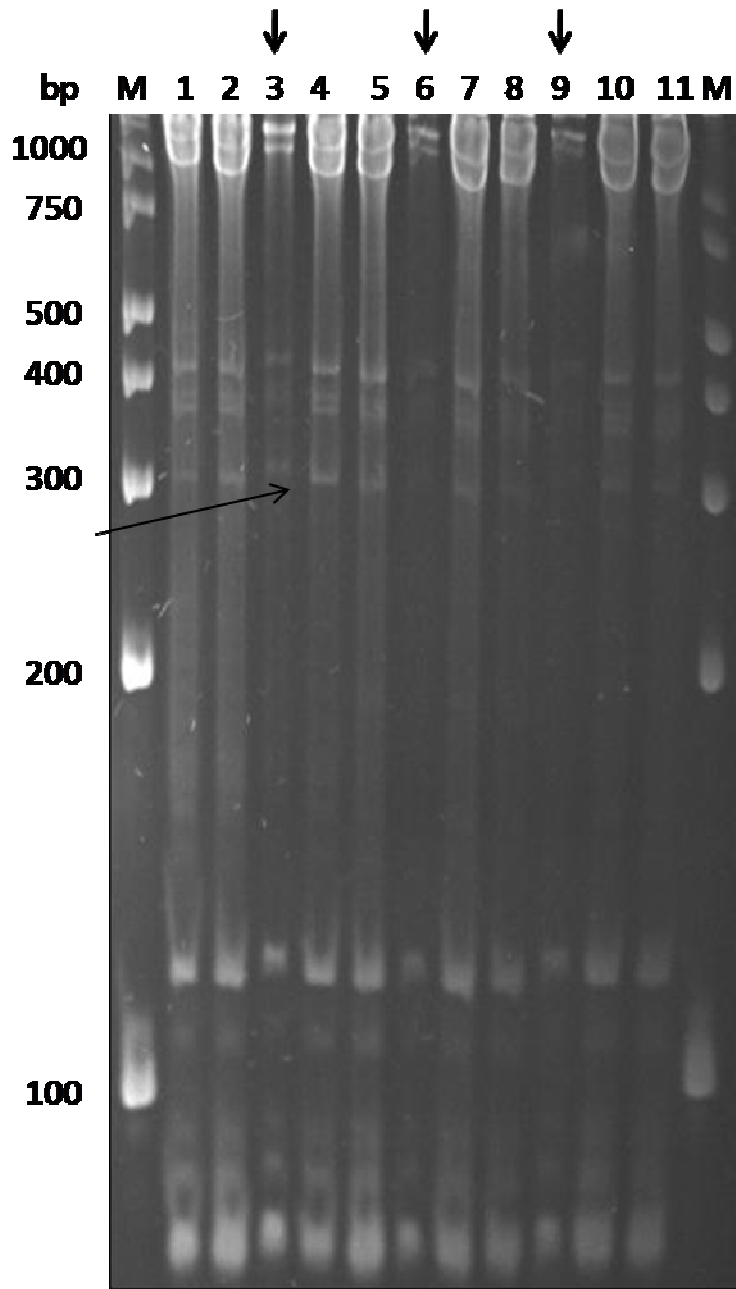
Figure 6: Gel picture comparing RNA isolated from different OD$_{600nm}$, both before and after rRNA removal. A selection of RNA samples with different OD$_{600nm}$ was run on a 5% denaturising PA gel for approximately 70 min at 45°C. Lane M contains RNA Century Marker-Plus. Lane 1: RNA isolated at OD$_{600nm}$=0.21. Lane 2: RNA isolated at OD$_{600nm}$=0.50. Lane 3: RNA isolated at OD$_{600nm}$=0.50 and treated with an mRNA purification kit. Lane 4: RNA isolated at OD$_{600nm}$=0.96. Lane 5: RNA isolated at OD$_{600nm}$=1.18. Lane 6: RNA isolated at OD$_{600nm}$=1.18 and treated with an mRNA purification kit. Lane 7: RNA isolated at OD$_{600nm}$=1.22. Lane 8: RNA isolated at OD$_{600nm}$=1.26. Lane 9: RNA isolated at OD$_{600nm}$=1.26 and treated with an mRNA purification kit. Lane 10: RNA isolated at OD$_{600nm}$=1.27. Lane 11: RNA isolated at OD$_{600nm}$=1.26 (after 32 h). The short arrows show RNA treated with an mRNA purification kit, whereas the longer arrow shows a slightly stronger band.

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Lanes 3, 6 and 9 on figure 6 contain RNA which was treated with rRNA-extracting magnetic beads. By comparing the intensities of bands representing rRNA extraction with untreated samples (e.g., lane 3 and lane 4), it is clear that large amount of rRNA was removed. Interestingly, lane 4 (OD_{600nm}=0.96) and lane 5 (OD_{600nm}=1.18) both contain a slightly stronger band at approximately 300 nt. This RNA is potentially interesting because it could indicate a higher expression of a particular RNA at this OD_{600nm}. All samples show a smear of RNA in the interesting area between 120-340 nt. Based on the growth curve (figure 4) and the gel on figure 6, I decided to construct a cDNA library based on RNA isolated from OD_{600nm}= 1.0. At this OD_{600nm}, the culture is in late exponential growth phase and the RNA is still in good shape (no detectable degradation).

3.1.3 Obtaining RNA in size range 120-340 nt

To construct a cDNA library from *V. salmonicida* cells at OD_{600nm}=1.0, a new culture of *V. salmonicida* was grown to OD_{600nm}=1.0 as described in section 2.2. A total of 600 µg total RNA was isolated using the Trizol protocol, treated with DNase, and size- fractioned on a 5% denaturising PA gel (figure 7).
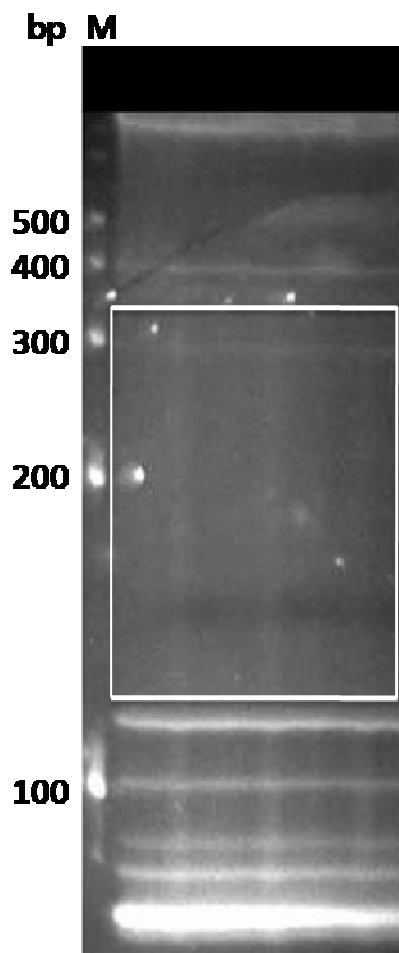
Figure 7: Gel picture of the size- fractionating PA gel. A total of 600 µg RNA isolated from *V. salmonicida* at OD_{600nm}=1.0 was run on a 5% denaturising PA gel for approximately 80 min at 45°C. The marked area was cut and the RNA was eluted.

The area indicated by an open box in figure 7, which represents RNA from approximately 120-340 nt, was cut from the gel. RNA was recovered from the gel by ON elution as described in section 2.8, and rRNA was removed by hybridization to RNA oligonucleotides attached to magnetic beads. A total yield of 5 µg RNA was obtained.

3.1.4. Full length cDNA production

The isolated, size fractionated, recovered and enriched RNA was next modified by adding poly(C)-tails to the 3' ends. Poly(C)-tailing was performed using Poly-A Polymerase as described in section 2.10, before a full length cDNA library was generated as follows: First, the RNA was treated with TAP to leave a phosphate at the 5' end of the RNA. Next, an RNA oligo was ligated onto the 5' end of the RNA, before a final reverse transcription of the RNA made the first strand of the cDNA library. The reverse transcription was carried out with a mixture of the 3 different oligonucleotides Oligo dC3'A, Oligo dC3'C, and Oligo dC3'T (see table 1 in appendix for sequence). The primers were customized to anneal to all C- tailed RNA. For details on making the cDNA library, see section 2.10. One µl samples were collected both before and after poly(C)-tailing, after TAP treatment and after Oligo ligation of the RNA. The samples were run on a 1% agarose gel (figure 8A.). The cDNA library was finally amplified by a touchdown PCR with primers GeneRacer 5' Primer and GeneRacer 3' Primer (see section 2.11.3 for details on cDNA amplification, and table 1 in appendix for primer sequences), parallel with two negative controls. Twenty µl of the amplified cDNA was run on a 1% agarose gel (figure 8B).
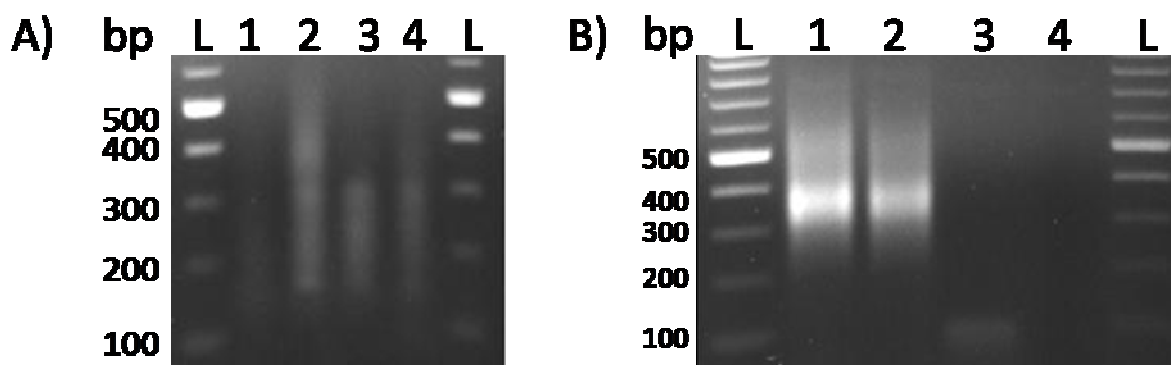
Figure 8: Samples taken during cDNA library production and after performing a Reverse Transcription to create the cDNA library. Both gels are 1 % agarose, run for 1 hour at 90 V. Image A) shows samples taken during the cDNA library production. Lane L contains 100 bp ladder, while lanes 1-4 contain RNA from both before and after Poly C-tailing, after TAP treatment and after Oligo ligation, respectively. Image B) shows results from amplification of the cDNA library. Lane L contains 100 bp ladder. Lane 1 and 2 contain 1 µl and 2 µl template, respectively. Lane 3 contains a negative template control, while lane 4 contains a negative primer control.

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In figure 8A, lane 1 contains size-fractionated RNA before poly(C)-tailing. A smear is expected between 120- 340 nt, and a weak smear is in fact visible in this area. Lane 2 shows a more distinct smear of larger RNA. This is consistent with poly(C)-tails being added to the RNA. In lane 3, the RNA had been treated with TAP. The decreasing smear can be caused a loss of RNA as a direct consequence of the phenol:chloroform extraction. After Oligo ligation, the smear represented RNA with slightly higher nucleotide size, which is consistent with the addition of a 44 nt RNA (lane 4).

Figure 8B shows PCR reactions amplifying the cDNA library. Lane 1 and 2 show reactions carried out with 1 and 2 µl template, respectively, and lanes 3 and 4 are negative controls. The negative controls are in fact negative, so a cDNA library was created. Four µl of the amplified cDNA library was cloned in to a gGEM-T Easy Vector and transformed into JM109 Competent Cells according to manufacturers' protocol. The whole transformation reaction was plated on several AMP plates, resulting in close to a thousand colonies.
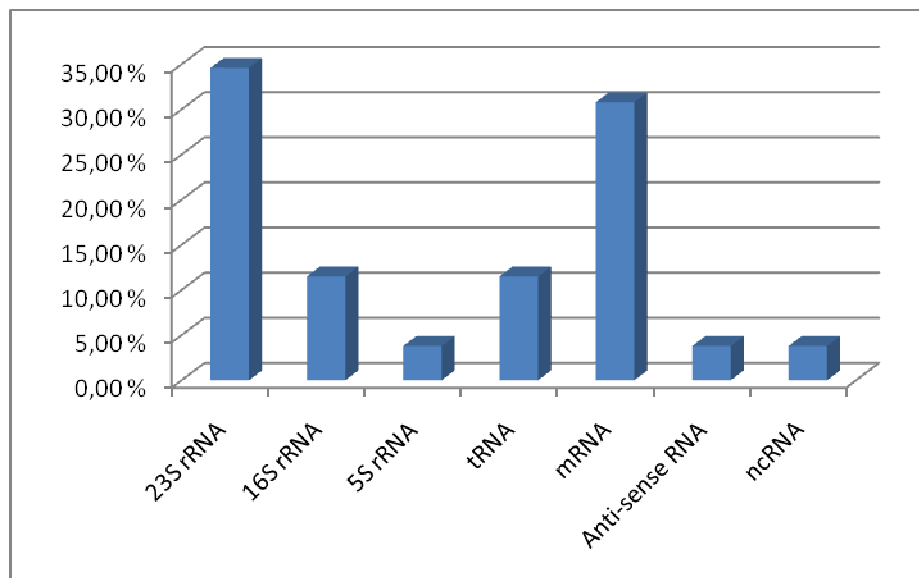
3.1.5   Initial analysis of the cDNA library

Sequencing of 44 random colonies was carried out to test the quality and relevance of inserts. The obtained sequences were viewed in Sequence Scanner v1.0 (figure 9) and all sequences were arranged in sense direction (that is 5'-Oligo-RNA-poly(C) tail-3'). As much as 20 percent of the sequenced clones gave a sequence quality so bad that it was impossible to obtain data from them, and another 20 percent turned out to have no insert in their vector. That leaves a total of 26 obtained sequences. The genomic viewer and annotation tool Artemis v.9 (Rutherford et al, 2000) was used to analyze the sequences. The whole genome of *V. salmonicida*, with both chromosomes annotated was available at the University of Tromsø (Hjerde et al, unpublished manuscript). A file with sRNA predictions for the whole genome, created by Rafi Ahmad (IMB, University of Tromsø, Norway), was also available. The computational predictions are based on homology to other known sRNAs and also typical locations of sRNAs (see figure 1 in introduction). With these tools at hand, an initial screen of the cDNA library started. The result from this screen is presented in figure 10.

25
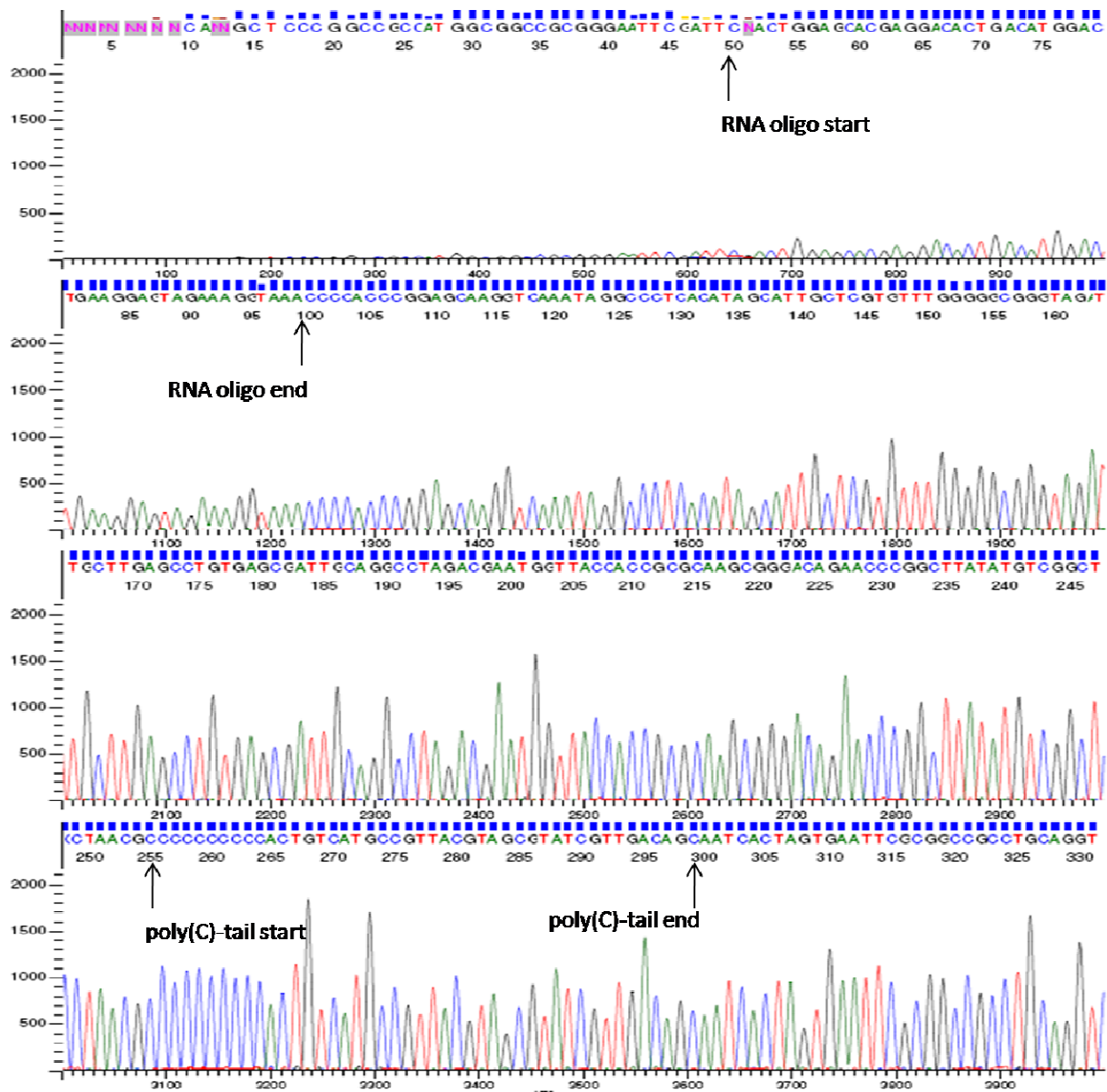
Figure 9: A sequence file viewed in Sequence Scanner. This image shows the sequence of Clone 40, viewed in Sequence Scanner v.1.0 (Applied Biosystems).

Figure 10: Pole diagram showing results from initial analysis of the cDNA library. The diagram shows the percent of the different RNAs obtained from sequencing of the cDNA library. Only 26 clones were sequenced, so the result does not reflect the actual percentages in the cDNA library.

The majority of the sequences belonged to rRNA (23S rRNA, 16S rRNA and 5S rRNA) and mRNA (figure 10). Two of the obtained sequences were of particular interest, named clone 38 and clone 40.

Figure 11: Two of the obtained sequences were of interest. The clones are presented in the order: 5'-Oligo-RNA-poly(C) tail-3'. The underlined nucleotides are the RNA oligo, the bold nucleotides are the poly(C)-tail, while sequence errors (when compared to the genome of *V. salmonicida*) are oversized.

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Both sequences were submitted to BLAST and Rfam but there were no specific hits. Clone 40 was a match to a predicted sRNA in Artemis, but the obtained sequence only had 160 nt, whereas the sRNA was predicted to be 337 nt long. The 337 nt sequence from *V. salmonicidas* genome was copied from Artemis and run through Rfam. This sequence was a match to the ribozyme RNaseP.

The second clone of interest was a sequence of 141 nt, on the anti-sense strand opposite a gene coding for Indole-3-glycerol phosphate synthase, which is involved in biosynthesis of amino acids (Kyoto Encyclopedia of Genes and Genomes (KEGG)). There is no sRNA predictions or predicted coding sequences in this area. The structures of clone 38 and 40 were predicted by MFOLD (Mathews et al, 1999; Zuker 2003) see figure 12.
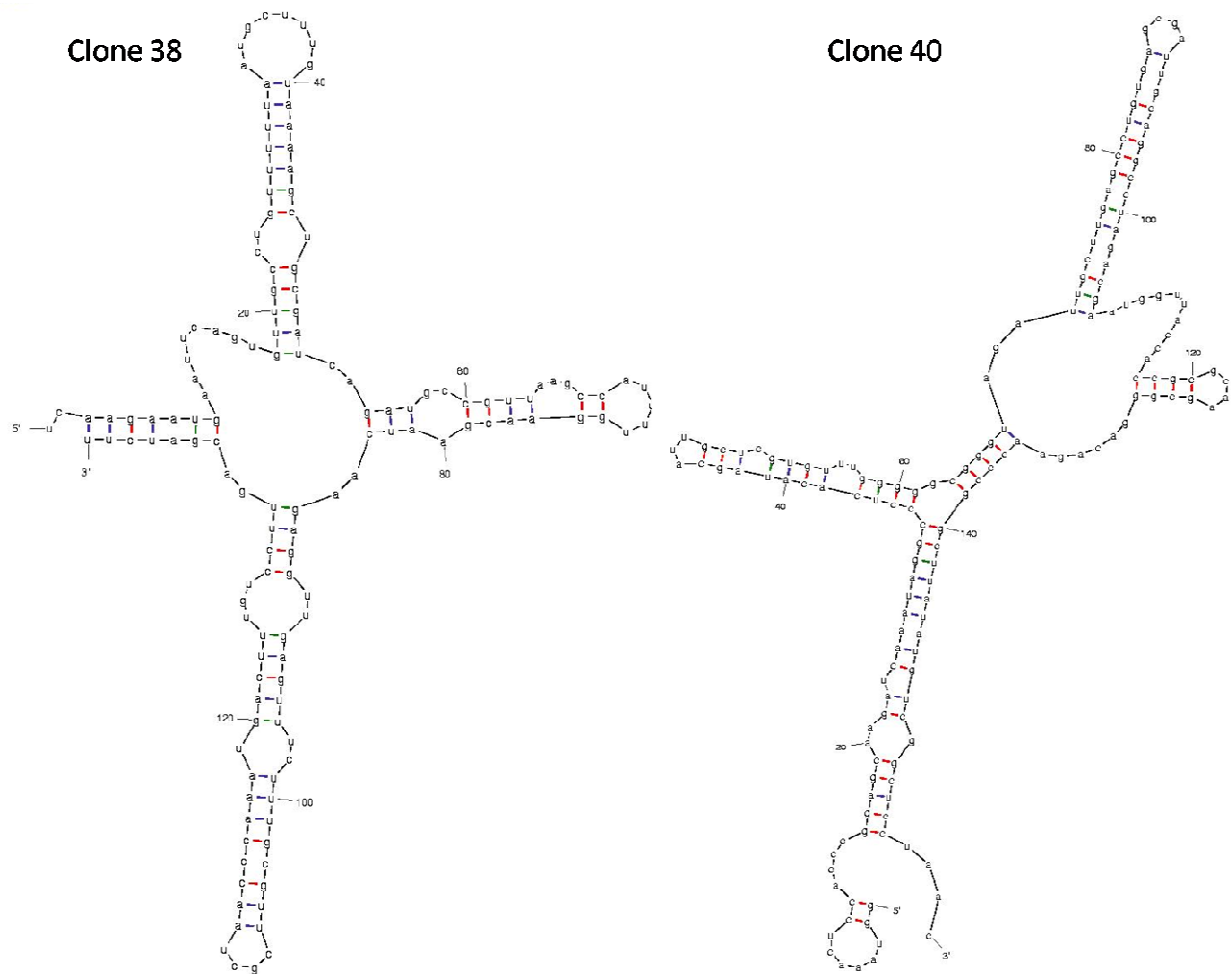
**Figure 12: Predicted secondary structures of Clone 38 and 40**. Possible secondary structures of two clones of interest, predicted by MFOLD (Mathews et al, 1999; Zuker 2003).

3.2 Design of a *qrr* knock-out construct

The second main task in this study was to make a *V. salmonicida qrr* deletion knock-out mutant. A knockout mutant is made in order determine the functional role of the gene(s) and study the effects on caused by the mutation. Expression of Qrr has earlier been confirmed in V. salmonicida by Northern blot analysis (PhD student G.Å. Hansen, unpublished results).

3.2.1 Design of primers for a *qrr* knock-out construct

A prerequisite for designing site-specific gene knock-out constructs is that the DNA sequence of the gene of interest and the surrounding region is available. The genome sequence of *V. salmonicida* was recently completed at The Wellcome Trust Sanger Institute, and the unpublished annotated genome is available at The University of Tromsø. The Artemis v.9 software (Rutherford et al, 2000) was used to explore the genome. The predicted sRNA gene *qrr* is 106 bp in length and located on the reverse (-) strand on *V. salmonicida*s chromosome 1. Adjacent genes should not be affected in the knock-out process. Four primers, named QrrA, QrrB, QrrC and QrrD were designed in order to generate the knock-out construct. Figure 13 shows primer locations.
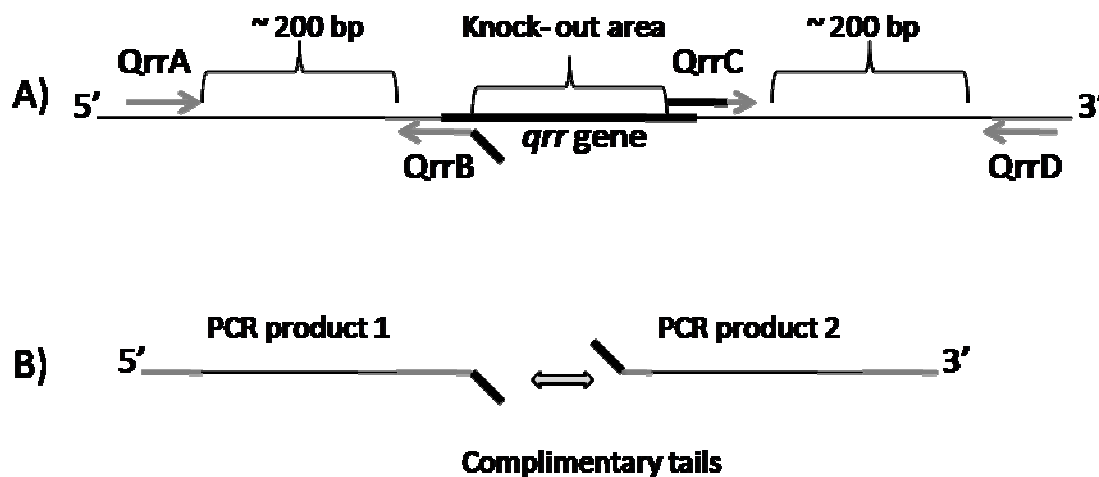

Figure 13: Illustration of the knock-out construct and primer locations. A) Illustrates the genome of *V. salmonicida* surrounding *qrr*. QrrA-D are primers designed in order to create a knock-out construct. B) Illustrates the complementary tails of the two PCR products (run with primers QrrA+QrrB and QrrC+QrrD) which will ensure their fusion.

To prevent reading frame shift, the number of bases in *qrr* that were to be deleted had to be dividable by three. Furthermore, QrrB and QrrC had to have complimentary tails, while QrrA and QrrD would need a recognition site in their 5' ends for the restriction enzymes *Xho*I and *Spe*I, respectively. At the 5' end of the restriction enzyme recognition sites, three extra nucleotides were added to accommodate optimal cleavage by the enzymes. The primer pairs QrrA-QrrB and QrrC-QrrD were each to amplify an area of approximately 200 bp on each side of the area to be deleted. After deciding a suitable area for primer design, the web site OligoPerfect™ Designer (Invitrogen) was used to design the primers. The suggested primer sequences were analyzed by Oligo Analyzer 3.1 (Integrated DNA Technologies) to avoid self- and heterodimers. The primers were designed to delete an area of 60 nt within *qrr* and to amplify an area of 253 nt upstreams from *qrr* and 225 nt downstreams of *qrr*. The primers were ordered from Operon, and are listed in table 1 in appendix.

3.2.2 Design of the *qrr* knock-out construct

In order to make a *qrr* knock-out construct, two separate PCR products had to be made. The two reactions were set up with primer pairs QrrA+QrrB and QrrC+QrrD, respectively, using the proofreading Phusion polymerase and total DNA from *V. salmonicida* as template (see section 2.11.1 for more details, and table 1 in appendix for primer sequences). Twenty µl from each PCR reaction was run on a 1% agarose gel to analyze the result (figure 14A).
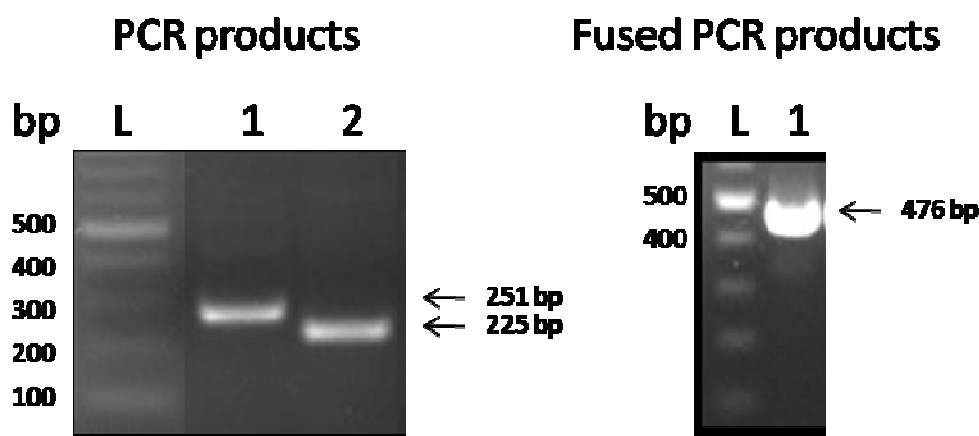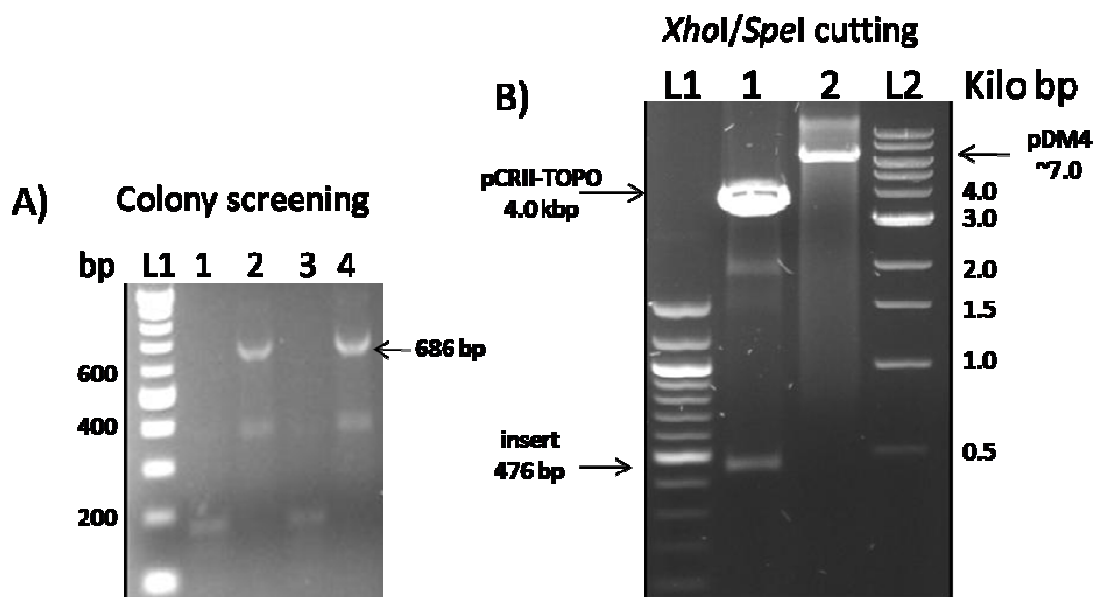
Figure 14: Verification of PCR products. PCR products were run on a 1% agarose gel for 1 h at 90 V. A) Lane L contains 100 bp ladder. Lane 1 contains primer QrrA+QrrB PCR products of 251 bp, while lane 2 contains primer QrrC+QrrD PCR products of 225 bp. B) Lane L contains 100 bp ladder, while line 1 contains the 476 bp fused product of the two PCRs on image A.

As expected, lane 1 on figure 14A shows a band of approximately 250 nt, whereas lane 2 shows a band of about 225 nt. In the next step the two PCR products were fused in a PCR, in the presence of Phusion polymerase as described in section 2.11.2. The product from the overlap PCR was run on a 1% agarose gel to confirm the size of the product. As figure 14B shows, the PCR resulted in a product of approximately 470 bp, which was cloned into a vector as described below.

3.2.3 Transformations and verifications

The ~470 bp fragment was next cloned into a pCRII-TOPO cloning vector. Before ligating the PCR product into the cloning vector the PCR product was treated with 1 U Taq polymerase and incubated at 72°C for ten minutes, to add A-overhangs (proofreading enzymes do not add A-overhangs). This cloning was done in order to make the final cloning of the insert into the suicide vector, named pDM4, easier. Cloning of PCR products directly into suicide vectors using restriction enzymes can be difficult, whereas subcloning of DNA fragments between vectors is less challenging. The pCRII-TOPO ligation reaction was transformed into One Shot DH5α competent cells, and colonies were screened using PCR and the universal primers M13F and M13R as described in section 2.11. The screening identified right sized inserts in the vector of two colonies (figure 15A).

Figure 15: Verification of insert in pCR-II Topo vector and restriction enzyme cutting. *E. coli* DH5α cells were transformed with a pCRII-TOPO vector containing overlap PCR product. A) Results from colony screenings with M13 F and M13 R primers. Expected size of positive PCR product is 686 bp. L1 contains 100 bp ladder. Lane 1 and 3 are negative for inserts, while lane 2 and 4 are positive. B) Cutting of plasmids with restriction enzymes *Xho*I and *Spe*I. L1 contains 100 bp ladder, while L2 contains 1kb ladder. Lane 1 shows cut pCRII-TOPO vector containing overlap PCR product. Lane 2 shows cut pDM4.

A colony containing the pCRII-TOPO vector with the expected insert was grown over night in the presence of AMP, and plasmids were isolated using the E.Z.N.A miniprep kit. Next, the insert was cut from the the pCRII-TOPO vector with restriction enzymes *Xho*I (cut site 5'-C^TCGAG- 3') and *Spe*I (cut site 5'-A^CTAGT- 3') (see section 2.16 for details). The pDM4 suicide plasmid was treated with the same enzymes, and products were run on a 1% agarose gel (figure 15B). The *Xho*I/*Spe*I-cut insert is in lane 1, whereas the *Xho*I/*Spe*I-cut suicide vector pDM4 is in lane 2. The cut products were extracted from the gel and mixed in a ligation reaction in a 3:1 insert-vector ratio (5 ng insert and 25 ng vector) (see section 2.17 for details on ligation reaction). The ligation mix was incubated at 16°C ON, and finally transformed into *E. coli* S17-1 cells (described in section 2.18). Colonies were screened by PCR using primer QrrA and QrrD, and run on a 1% agarose gel (figure 16A).

33
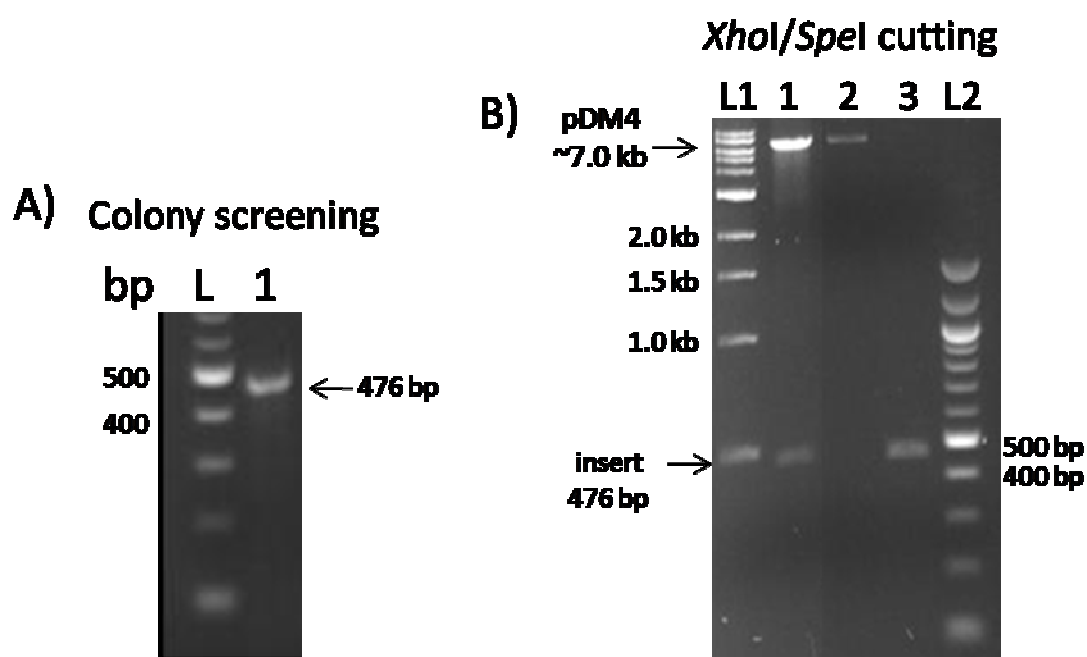
Figure 16: A) Screening of *E. coli* S17-1 cells using QrrA and QrrD. B) Verification of insert. L1 contains 1kb ladder. Lane 1 contains purified and cut suicide vector pDM4 isolated from *E. coli* S17-1 cells. Lane 2 show a control, where pDM4 has not been cut. Lane 3 is a control containing only the qrr knock-out overlap construct. L2 contains 100 bp ladder.

Figure 16A shows one clone that contains the expected insert of ~470 bp. An ON culture of this clone was grown in the presence of CAM (see section 2.13 for details) and the pDM4 vector was isolated. The vector was cut with *Xho*I and *Spe*I to verify the right sized insert (see section 2.16 for details). Figure 16B shows the image of a 1% agarose gel run after the cutting. Lane 1 contains the cut vector, while lane 2 and 3 are positive controls for the pDM4 vector (empty vector) and the insert (gel-purified insert), respectively. This confirms that the pDM4 vector contains the right sized insert. The insert was sequenced using primers QrrA and QrrD, to verify the sequence and the transition between the insert and the suicide vector pDM4. The result showed no errors in the sequence, and the construct was named pDM4-qrr. pDM4-qrr was finally transformed into *E. coli* β-2155 DAP auxotroph cells (see section 2.18 for details). These cells were used for conjugation purposes.

3.2.4 Conjugation of pDM4-qrr into *V. salmonicida*

To conjugate the pDM4-qrr plasmid from *E. coli* β-2155 to *V. salmonicida* cells two separate ON cultures were grown, one with *V. salmonicida* LFI1238 and the other with pDM4-qrr-containing DAP auxotroph *E. coli* β-2155 cells. Next day, new cultures were inoculated to $OD_{600nm}\sim0.1$-0.2. The cultures were grown to exponential phase (approximately $OD_{600nm}\sim0.6$), mixed and transferred as spots to a LA plate containing DAP. Plates were first incubated at RT and then placed at 12°C. The spots were resuspended and plated on LA plates containing a low concentration of CAM. These plates were incubated at 12°C for 3-5 days before they were examined for small, transparent colonies that would indicate a *V. salmonicida* transconjugant. Potential transconjugants were picked and spread on LA plates with CAM and again incubated at 12°C for 3-5 days. Several conjugations were carried out without any positive results.

4.0   Discussion

In a bigger perspective, this study is part of an ongoing project which aims to use *V. salmonicida* as a model organism for the study of virulence, host specificity and adaptation, as well as expression of cold adapted proteins. Specifically, this study is a part of an effort to understand the roles of small regulatory RNAs (or sRNAs) in *V. salmonicida*s virulence.

4.1   Analyses of the cDNA library

Due to time limitations, only 44 vectors obtained from clones were sequenced and 26 inserts were analysed. Sequencing of vectors with no inserts could have been prevented by adding X-gal/IPTG to plates before growing the transformants. However, by adding IPTG, cloned small RNAs will be expressed as part of the lacZ mRNA and can potentially be toxic to *E. coli* All sequencing reactions were run in parallel, but as much as twenty percent of the sequences were of bad quality. Because of the good quality of the obtained sequences, this is not likely to be caused by reagents or the PCR program, but factors like too low concentration of template in the reaction (only a random selection of samples were measured by spectrometry before reagents were calculated). A previous attempt to make an sRNA library from *V. salmonicida* resulted in approximately 60% rRNA, so an rRNA removal step was added. Because of the low number of sequences, it is not possible to make a decision regarding the quality of the cDNA library or to know if the rRNA removal step was a success. A new sequencing of close to 200 transformed colonies will provide a better foundation for further decision making. This will make it possible to find the relative frequency of sRNAs in the library.

4.2   Optimization of conjugation to obtain transconjugants

This was an attempt to make a knock-out by deletion. A knock-out by insertion is a less time consuming method, and would maybe in a short time-frame like this have been an easier goal to achieve. On the other hand, a deletion knock-out is of greater scientific value once it is generated.

The conjugation method used in this study has been established in Prof. Debra Milton's (University of Umeå, Sweden) laboratory for *V. anguillarum* (Milton et al, 1995), and is not an established method for *V. salmonicida*. *V. anguillarum* was in Miltons study grown at RT, whereas the optimum temperature for cultivation of *V. salmonicida* is ~15°C. Therefore, the challenge lies in transferring this method to a psychrophilic bacterium like *V. salmonicida*. The conjugation process itself was here carried out at RT for 7 h. Lowering the temperature might work better for *V. salmonicida* without affecting the ability for *E. coli* to form its phili, and mate.

4.3   Further research

To ensure the quality of the cDNA library, more clones need to be sequenced. A good quality library can be used in future ultra-high throughput DNA sequencing studies. An example of such an approach is the 454 sequencing system (http://www.454.com), where as much as 400.000 reads can be obtained in parallel. This will provide a large amount of information, and chances of discovering novel sRNAs are very high.

In this study, a cDNA library was constructed from OD_{600nm}=1.0. Another possibility is to create cDNA libraries from different OD_{600nm} values in order to investigate sRNA expression at different phases on a growth curve. Creating more cDNA libraries from different growth phases and pool them will make it possible to investigate total sRNA expression. It is also possible to stress bacterial cultures with conditions like high iron concentration or high/low temperature and compare these cDNA libraries to a library from a control culture.

Discovery of novel sRNAs can open for further experimental approaches to confirm gene expression and function. Northern blot analysis can be used to confirm gene expression and enzyme assays can give more insight about the sRNAs function in *V. salmonicida*. Functions of novel sRNAs can be investigated by designing a knock-out mutant.

As for the *qrr* knock-out construct, this can be used in further conjugation attempts. A knock-out of *qrr* in *V. salmonicida* is particularly interesting because the bacterium holds only one known copy of the Qrr encoding gene, whereas other *Vibrio*s like *V. harveyi* has five and *V. cholerae* has four identified Qrrs that cooperate (Tu and Bassler, 2006; Lenz et al, 2004). An effort to optimize the conjugation process for V. salmonicida is in progress. The challenge lies in finding a balance between the optimal temperature of both the recipient and the donor.

A *qrr* knock-out mutant can be used in further experiments in order to reveal more about Qrrs function in *V. salmonicida*. A Northern blot analysis will be able to tell if expression of *qrr* is turned off. The knock-out mutant can be compared to a control culture in microarray experiments to identify genes that are turned on/off in the presence and absence of Qrr. Expression experiments can be run in parallel with an overexpressed control culture, where qrr is constantly expressed.

The functional diversity of novel regulatory RNAs in bacteria reveals the importance of further studies to get new insights in growth, virulence, stress adaptation and possible targets for new antibiotics.

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6 Appendix

Reagents, solutions and growth medium:

SOC medium	**1 % LA plates**	**1% LB**
20 g Bactor Tryptone	10 g Bacto Peptone	10 g Bacto Peptone
5 g Bacto yeast extract	5 g Bacto yeast extract	5 g Bacto yeast extract
0.5 g NaCl	10 g NaCl[1]	10 g NaCl[1]
10 ml 250 mM KCl	16 g Agar	dH$_2$O to 1 litre
dH$_2$O to 1 litre	dH$_2$O to 1 litre	

All mediums were adjusted to pH 7.5 and autoclaved.

[1]Plates and medium for *V. salmonicida* were added 25 g NaCl, for a final concentration of 2.5 %.

10X TBE	**10X TAE**
108 g Tris base	48.4 g Tris base
55 g Boric acid	11.4 ml Glacial Acetic Acid
9.3 g EDTA	3.7 g EDTA
MQ- water to 1 litre[2]	MQ Water to 1 litre

[2]10x TBE used in RNA gel was added DEPC- water and autoclaved before use.

Antibiotic and DAP stocks:

10 µg/ml Ampicillin (AMP)

10 µg/ml Chloramphenicol (CAM)

150 mM 2,6-Diaminopimelic acid (DAP)

5 % denaturating PA gel	**Stop solution**
15.0 ml 40 % acrylamide	19 ml Formamide
12.0 ml 10X TBE buffer	800 µl EDTA, 0.5 M, ph 8.0
57.6 g Urea	200 µl DEPC- water
55.0 ml DEPC- water	0.01 g Bromophenyl blue
Stirred until urea was dissolved, then added	0.01 Xylenecyanol
90 µl Temed ans	
900 µl 10 % ammonium persulphate	
and set in the chamber	

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Table 1: Oligonucleotides used in this study. Primers named Qrr were used to create the knock-out construct, and the following applies for them; the grey areas show complimentary tails, the bold areas show restriction enzyme cut sites, while the underlined areas are randomly added nucleotides.

Oligonucleotide name	Sequence (5'-3')
Qrr A forward primer	TAA**CTCGAG**CGATAAAGCGCAGCAACA
Qrr B reverse primer	AACCGTAATATACCGCCTTTGGCTTAAAGGGTC
Qrr C forward primer	CGGTATATTACGGTTGGCTTC
Qrr D reverse primer	CGA**ACTAGT**AAGAAGGAGCGAGTTATCAATC
M13 forward	GTTTTCCCAGTCACGAC
M13 reverse	CAGGAAACAGCTATGAC
Oligo dC3' A	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGGGGGGGGGGA
Oligo dC3' C	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGGGGGGGGGGC
Oligo dC3' T	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGGGGGGGGGGT
GeneRacer™ 5' Primer	CGACTGGAGCACGAGGACACTGA
GeneRacer™ 3' Primer	GCTGTCAACGATACGCTACGTAACG

The below sequence shows *qrr* (highlighted in grey) and its surrounding area. Primers were designed to amplify two areas; one upstreams of *qrr* and one downstreams. Primer sequences are underlined and shown in bold letters in the following order: QrrA, QrrB, QrrC and QrrD. The area between primer QrrB and QrrC is the deleted area. The sequence is shown in 5'-3' direction.

AATTAAATCCGGTGCCTTTAACGTCACTTTTTCAATCGCATCAACACCTTTTGCTACAACATCGATATCTAAACCT
AAAGGATTAAGGTAAGAA**CGATAAAGCGCAGCAACA**GAAGCGGTGTCTTCAACCATGAGTAAATATTTTTTTTG
CATCAGAATCCAAACCATTAAAAAGCATATCAACCTTTGCATTTTCGCATAATGCAATTGCATAACGCAAAGATA
TTTTCGCATTATGCAAAACATTTGTGATATCGCGTCTTTTTTGTACTAAATTAGTCGTTTTTCAGGCTATGCAAA
GTTGGCACGCTCTCTGCTATATGTATAG**TGACCCTTTAAGCCAAAGG**GTCACCTAGCCAACTGACGTTGTTAGTG
AAATTTACTTTCACATGAACAATAAAGCCAAC**CGGTATATTACGGTTGGCTTC**TTTTTTCTTAAAATCAATAAT
TTACAAAATAGTCATTAACTATTCGTTATAAA'TTGATTCCTTAATTTCTCTACCTCATCCCTTACTTTAGCAGCC
TGTTCAAACTCTAGATCTCTCGCATGTTGATACATTTTTGCTTCTAATCGTTGAACTTCTTTTTCTAATTGCTGCG
GCGTCATC**GATTGATAACTCGCTCCTTCTT**CTGCCACTTCTGATAGCTTCATTTTAGGTGCGACTATTTTCCGTT
TATTCTTAGTCATATCCCCCAATTCAAGAATATCAGCCACATTTTTTTTCAATGCGGTT