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A genome-wide study of gene expression regulation in the Gram-negative fish pathogen *Aliivibrio salmonicida*

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A dissertation for the degree of Philosophiae Doctor

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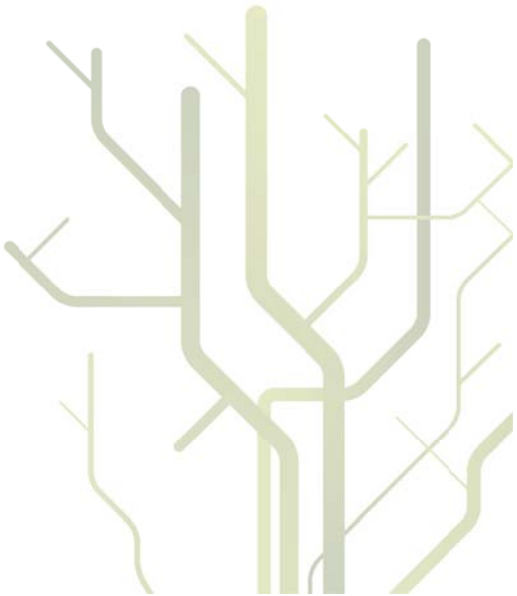

To Mille and Marthe

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Tromsø, May 2011

Geir Åsmund Hansen

Abstract

Regulation of gene expression is essential for all bacteria to function and survive. The expression of genes into proteins is dependent on a number of steps, each of which can be subjected to regulation. In the literature, much emphasis has typically been placed on transcriptional regulation, but gene regulation at the post-transcriptional level has also gained much attention during recent years. One contributing factor to this shift in knowledge is the accumulating amount of genomic data that has become available, and computational methods now play important roles in mapping genomic features, such as promoters and transcription factor binding sites.

In the present work, the Gram-negative fish pathogen *Aliivibrio salmonicida* has been used as a model for computational genome-wide predictions of transcription factor binding sites and small regulatory RNAs. In **Paper 1**, a bioinformatical search for Ferric uptake regulator transcription factor binding sites resulted in the identification of several genes previously not known to be regulated by the Ferric uptake regulator. The prediction and validation of genes that encode small regulatory RNAs in **Paper 2** increased the knowledge on the non-coding RNA pool of our model system. **Paper 3** and **Paper 4** provide in-depth studies on two of the identified small regulatory RNAs from **Paper 2**, the Spot 42 homolog and the novel VSsrna24.

Overall, the results from these studies have increased our general understanding on gene regulation in *A. salmonicida*. In addition, the work has generated a number of good starting points for future experiments.

List of papers

Paper 1

Rafi Ahmad, Erik Hjerde, Geir Åsmund Hansen, Peik Haugen, Nils-Peder Willassen (2008). **Prediction and Experimental Testing of Ferric Uptake Regulator Regulons in Vibrios.** *J Mol Microbiol Biotechnol*. 16:159-68.

Paper 2

Rafi Ahmad, Geir Åsmund Hansen, Hilde Hansen, Erik Hjerde, May Liss Julianne Nyrud, Nils-Peder Willassen, Peik Haugen (2010). **Prediction, microarray, and Northern blot analyses identify new intergenic small RNAs in *Aliivibrio salmonicida*.** Under review, *PLoS ONE*.

Paper 3

Geir Åsmund Hansen, Rafi Ahmad, Erik Hjerde, Christopher G. Fenton, Nils-Peder Willassen, Peik Haugen (2011). **Expression profiling of a *spf* deletion mutant suggests biological roles and mRNA targets for Spot 42 in the fish pathogen *Aliivibrio salmonicida*.** Submitted to *RNA Biology*.

Paper 4

Geir Åsmund Hansen, Rafi Ahmad, Erik Hjerde, Christopher G. Fenton, Nils-Peder Willassen, Peik Haugen (2011). ***Aliivibrio salmonicida* encodes a small RNA immediately downstream of *spf*: Microarray analysis and potential roles in carbohydrate transport and metabolism.** Manuscript (for submission to *PLoS ONE*).

Abbreviations

ATP – Adenosine triphosphate

cAMP – Cyclic adenosine monophosphate

CCR – Carbon catabolite repressor

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CRP – cAMP receptor protein

DNA – Deoxyribonucleic acid

EI/EII – Enzyme I/II of the PTS system

ETC – Electron transport chain

Fur – Ferric uptake regulator

Hpr – histidine phosphocarrier protein

IGR – Intergenic region

mRNA – Messenger RNA

NADH – Nicotinamide adenine dinucleotide

ncRNA – Non-coding RNA

ORF – Open reading frame

PDH – Pyruvate dehydrogenase complex

PEP – Phosphoenolpyruvate

PTS – Phosphoenolpyruvate phosphotransferase system

RNA – Ribonucleic acid

RNaseP – Ribonuclease P

rRNA – Ribosomal RNA

sRNA – Small RNA

TCA – Tricarboxylic acid cycle

TF – Transcription factor

TFBS – Transcription factor binding site

tmRNA – Transfer-messenger RNA

tRNA – Transfer RNA

TU – Transcriptional unit

UTR – Untranslated region

I. Introduction

1. Regulation of bacterial gene expression

In order to function and survive, all bacteria need to sense and respond to changes in their environment (Boor, 2006). Through regulation of gene expression, the cells are able to adapt to changing growth conditions and new surroundings. Complex regulatory networks make the cells capable of converting environmental stimuli into fine-tuned global changes in gene expression (Beisel and Storz, 2010). These important changes allow bacteria to efficiently utilize available nutrients and to resist chemical and biological harmful substances that are present in their surroundings.

The expression of genes into products requires several steps, all of which in bacteria are exploited to exercise control of gene expression. Regulation of transcriptional initiation is the "classical" example of how genes are turned on and off and serves as an important and efficient regulatory step that controls the transcription of genes into RNA (reviewed by Browning and Busby, 2004). However, during recent years, post-transcriptional gene regulation has attracted more attention as it has become evident that a significant part of the total expression regulation takes place at the RNA level (i.e., post- transcriptionally).

1.1. Transcriptional regulation

Transcriptional regulation affects the production of RNA transcripts from specific DNA transcriptional units (TUs). In a bacterial genome, a TU constitutes a regulatory region, a transcription start site, one or more open reading frames (ORFs), and a transcription termination site (Balleza et al., 2009). The TU might be monocistronic or polycistronic, i.e., depending on if they contain one or more ORFs, respectively. In prokaryotes, the regulatory region of the TU can be up to 400 base pairs in length and contain *cis* elements such as promoters and transcription factor binding sites (TFBSs) (Collado-Vides et al., 1991). During transcription initiation in bacteria, an RNA polymerase, in association with a sigma factor, is required for proper promoter recognition (Maeda et al., 2000). Binding of this complex to the promoter region might be modulated by transcription

factors (TFs) bound to TFBSs (Browning and Busby, 2004). To function as regulatory switches, the TFs require the ability to both interact with TFBSs and to function as a signal sensor by ligand-binding or protein-protein interactions (Goulian, 2004). Negative regulators typically bind inside the promoter regions, thereby interfering with RNA polymerase, whereas the positive regulators bind upstream of the promoter regions to promote RNA polymerase binding (Collado-Vides et al., 1991; Madan Babu and Teichmann, 2003). Regulations by TFs are complex and dependent on many factors. For example, the regulatory region of a TU can be occupied by several TFBSs, different TFBSs are able to recruit the same TF, different TFs can recognize similar sites, and regulation is dependent on TF concentration and TF-TFBS affinity (Balleza et al., 2009). Generally, global TFs are less specific and must be expressed at higher levels compared to local TFs (Lozada-Chavez et al., 2008; Martínez-Antonio et al., 2008).

1.2. Classes of RNA and post-transcriptional regulation

Since the beginning of this millennium, it has become evident that important gene regulatory steps take place post-transcriptionally (Nogueira and Springer, 2000), i.e., at the RNA level. The world of RNAs can be separated into two main classes: messenger RNAs (mRNAs) serving as the protein coding template responsible for carrying information from the genes to the ribosomes and the non-protein coding RNAs (ncRNA) involved in different cellular processes. Bacteria contain a diverse set of ncRNAs transcribed from 10-15 percent of their compact genomes (Westhof, 2010). rRNAs, tRNAs, RNaseP, and tmRNA serve “housekeeping” functions in the cell, CRISPR RNAs acts as a prokaryotic immune system protecting the cell from viruses and plasmids (Horvath and Barrangou, 2010), whereas other classes of ncRNAs act as regulators of gene expression.

Riboswitches are *cis*-encoded RNA regulators located in the 5′ untranslated region (UTR) of mRNAs that change the expression of the corresponding mRNA by binding to small metabolites (Roth and Breaker, 2009). Some regulatory RNAs are located on the opposite strand of protein coding regions (i.e., anti-sense RNAs), whereas other regulatory RNAs are transcribed in *trans* from separate transcription units located in the intergenic regions (IGRs) (the region in between genes) of the genome.

1.2.1. Bacterial small regulatory RNAs (sRNAs)

The *trans*-encoded regulatory RNAs in bacteria are small in size (generally 50-200 nt (Huang et al., 2009) – hence the name small RNAs, or sRNAs) and modulate the expression of their targets (Gottesman, 2005; Waters and Storz, 2009). They have been reported to affect cellular processes like iron homeostasis (Massé 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). The first sRNAs were discovered in *Escherichia coli* three decades ago (Griffin, 1971; Ikemura and Dahlberg, 1973), but their abundance and important functional roles have been accepted only recently. By the end of the last century, only 10 sRNAs were known in *E. coli*, but this number quickly increased when a number of systematic genome-wide searches were started (Argaman et al., 2001; Rivas et al., 2001; Wassarman et al., 2001; Chen et al., 2002). These initial pioneering studies led to a series of similar works in other, mostly pathogenic, species, resulting in a growing list of sRNAs or potential sRNAs. The studied species include, e.g., *Bacillus subtilis* (Irnov et al., 2010), *Vibrio cholerae* (Livny et al., 2005; Liu et al, 2009), *Pseudomonas aeruginosa* (Livny et al., 2006), *Staphylococcus aureus* (Pichon and Felden, 2005), and *Listeria monocytogenes* (Mandin et al., 2007). According to the sRNAMap database (Huang et al., 2009), 79 sRNAs have been identified in *E. coli* strain K12 MG1655 (87 in all *E. coli*), and a total of 397 sRNAs have been identified in 28 bacterial species as of May 2011. Several new approaches, such as bioinformatic predictions, microarray, RNA-seq, Hfq co-immunoprecipitation, and RNomics, have shown to be valuable tools in genome-wide sRNA searches (Sharma and Vogel, 2009), giving future studies the possibility to discover even more sRNAs.

1.2.2. sRNA regulations

Most characterized sRNAs act as regulators by targeting mRNAs by imperfect sequence complementarities, generally at the 5' UTR, causing either a repression or activation of translation (Storz et al., 2004; Gottesman, 2005; Waters and Storz, 2009). Repression can either be caused by a nuclease-mediated cleavage of the mRNA, e.g., RNase E cleavage

of RyhB-*sodB* complex during regulation of iron homeostasis (Massé et al., 2003), or by blockage of ribosome entry, e.g., Spot 42 binding to *galETKM* causing discoordinate expression of the genes in the operon (Møller et al., 2002a). Activation can be caused by a change in the secondary structure, e.g., DsrA binding *rpoS* mRNA changes the mRNA structure leading to exposure of the ribosome binding site (Majdalani et al., 1998). sRNAs can also bind directly to proteins, thereby modulating the activities of the proteins directly, e.g., CsrB and CsrC binding to CsrA, antagonizing its activity (Liu et al., 1997, Weilbacher et al., 2003). In addition, many sRNAs require the bacterial conserved RNA chaperone Hfq to function (Valentin-Hansen et al., 2004). Hfq resembles eukaryotic Sm proteins in structure and function by stabilizing RNA-RNA interactions (Zhang et al., 2003). It has been proposed that Hfq binds an A/U rich stretch within the sRNA and target mRNA (Møller et al., 2002b; Zhang et al., 2002). Interestingly, Wadler and Vanderpool (2007) have reported a dual function ncRNA encoding both an sRNA and a protein. The sRNA SgrS, involved in the repression of glucose transporters of the phosphoenolpyruvate phosphotransferase system (PTS), also encodes a small protein, SgrT, which inhibits glucose transport by another mechanism than its sRNA precursor.

Hfq-sRNA complexes are involved in many regulatory networks in *E. coli* (Beisel and Storz, 2010). Typically, the networks include a protein TF directly sensing a biological signal or a two-component system responding to environmental stimuli. The sRNA component(s) of the regulon might be expressed during conditions such as anaerobic growth [Fnr activates FnrS (Boysen et al., 2010; Durand and Storz, 2010)], oxidative stress [OxyR activates OxyS (Altuvia et al., 1997)], glucose availability [CRP represses Spot 42 (Polayes et al., 1988)], iron availability [Fur represses RyhB (Massé and Gottesman, 2002)], and osmotic imbalance [EnvZ-OmpR activates MicF (Takayanagi et al., 1991)]. Most of the Hfq-binding sRNAs act through a single input module to repress genes in response to a particular environmental stimulus (Beisel and Storz, 2010). Such regulations involve a single regulator which co-ordinately modulates the expression of multiple genes, with none of the target genes regulating each other. The effect of the sRNA can often reverse the relationship between the protein TF sensing a biological signal and its target mRNA(s). This can be seen in the regulation of iron homeostasis where the sRNA RyhB turns the TF *Ferric uptake regulator* (Fur) into an

indirect activator (Beisel and Storz, 2010). RyhB negatively regulates the expression of many iron-containing proteins and is itself repressed by Fur. The net outcome is positive regulation when iron is abundant.

1.2.3. Computational sRNA predictions

During the last decade, a number of larger screens for sRNAs have been carried out in bacteria. These studies have resulted in a still growing list of sRNA candidates with a variety of different functions and mechanisms of work (reviewed by Backofen and Hess, 2010). Up to 2001, only 10 sRNAs were known in *E. coli* (Wassarmann et al., 1999), but from then, a number of genome-wide searches, both in *E. coli* and other bacteria, have led to a large increase in potential transcripts. It has been proposed that enterobacteria with 4-5 Mb genomes contain around five percent sRNAs compared to protein-coding genes, corresponding to 200-300 sRNA genes (Vogel and Wagner, 2007). In *E. coli*, the 80 identified sRNAs make up ~2 percent of the number of protein-coding genes, but this number has been suggested to represent only half of the actual sRNAs present in the genome (Zhang et al., 2004; Tjaden et al., 2006; Vogel and Wagner, 2007).

The first genome-wide sRNA searches were carried out at the start of the millennium using systematic bioinformatic approaches (Argaman et al., 2001; Rivas et al., 2001; Wassarman et al., 2001; Chen et al., 2002). Current approaches usually utilize a combination of conserved features from known sRNAs, including location (most sRNAs identified so far are located in the IGRs of the genome), putative promoters (e.g., sigma factors), TFBSs, transcription terminators, conservation of sequence, conservation of synteny of flanking genes in closely related species, and conservation of secondary structure motifs.

Compared to computer-based identification of protein-coding genes, computational prediction of sRNA genes involves a number of challenges. The sRNAs are shorter and their primary structure is usually not as important for the function as their secondary structure (Huttenofer et al., 2005), leaving the sRNA genes difficult to predict solely based on sequence similarities (Livny and Waldor, 2007). In general, the sRNA genes are reasonably conserved between closely related species, but they might be absent or more divergent in sequence between distantly related species. Another issue is that the

sRNAs are not translated into peptides and are therefore lacking features usually utilized in prediction of protein-coding genes (i.e., start and stop codons, open reading frames, etc.). Studies of promoters and TFBSs are usually restricted to *E. coli*, making the results difficult to apply to distantly related bacteria which usually require species-specific consensus sequences.

1.3. Regulation of major cellular processes occur both at the transcriptional and post-transcriptional levels: Two examples from the literature

The existence of all living organisms is dependent on availability of iron and carbohydrates. Bacteria have developed tightly regulated systems for the uptake and utilization of these substances, and regulation of iron homeostasis and uptake and metabolism of carbohydrates serve as excellent examples of regulons that use both transcriptional regulation and post-transcriptional regulation of gene expression as part of their regulation circuits. Both examples have been investigates in detail during this study, and central gene regulation steps will therefore be described in further detail in the following sections.

1.3.1. Regulation of carbohydrate metabolism

Bacteria that utilize organic compounds both as a source for carbon and energy are categorized as chemoheterotrophs. Faced with an environment where multiple carbon sources are available, bacteria will effectively select preferred sugars over non-preferred. This regulation is termed carbon catabolite repression (CCR) and affects as many as 5-10 percent of all bacterial genes (reviewed by Görke and Stülke, 2008). Glucose is the preferred carbon source in the majority of model bacteria. When present in the immediate surroundings, the bacterium will metabolize glucose, while genes that enable utilization of secondary substrates are not expressed and therefore inactive (Contesse et al., 1969). The ability to select the carbon source which will allow fast growth is important for bacteria in order to compete for limited resources in natural environments (Görke and Stülke, 2008).

An important part of CCR relies on the phosphoenolpyruvate (PEP)-dependent PTS system, a major mechanism for uptake of carbohydrates in bacteria. First described

in *E. coli* by Kundig et al. (1964), the PTS system consists of a membrane-bound sugar specific permease, enzyme II (EII), which together with two cytoplasmic general components, enzyme I (EI) and histidine phosphocarrier protein (HPr), make up the PEP-driven uptake and concomitant phosphorylation of numerous carbohydrates. The sugar specificity of the PTS system resides in EII, and bacteria usually contain a whole set of EIIs. At least 15 EIIs have been found in *E. coli*, and a similar number is present in *B. subtilis* (Deutscher et al., 2006; Deutscher et al., 2002; Reizer et al., 1999). The EII complexes are either a single multidomain protein or are formed by distinct proteins. The main role of the three PTS components is to transfer a phosphoryl group from PEP to the carbohydrates taken up by the cell.

In *E. coli*, the PTS general components, EI and HPr, are encoded by *ptsI* and *ptsH*, respectively (reviewed by Deutscher et al., 2006). The glucose specific EII complex is made up by two distinct proteins, the *crr*-encoded cytoplasmic $EIIA^{Glc}$ and the *ptsG*-encoded membrane-bound $EIIBC^{Glc}$. *ptsI, ptsH*, and *crr* together make up an operon (de Reuse et al., 1985). $EIIA^{Glc}$ functions as the master regulator of CCR in *Enterobacteriaceae* through interplay between CRP (cAMP regulatory protein), adenylate cyclase, and cAMP (Deutscher et al., 2006). When glucose is available as a carbon source, $EIIA^{Glc}$ is predominantly dephosphorylated (Hogema et al., 1998). Dephosphorylated $EIIA^{Glc}$ interacts with several non-PTS permeases to inhibit their activity in a process called inducer exclusion (Nelson et al., 1983; Osumi and Saier, 1982; Dills et al., 1982; Sondej et al., 2002). In addition, adenylate cyclase catalyses the conversion of ATP into cAMP and is activated by phosphorylated $EIIA^{Glc}$ (Feucht and Saier, 1980; Reddy and Kamireddi, 1998). Thus, glucose growth leads to low intracellular concentration of cAMP.

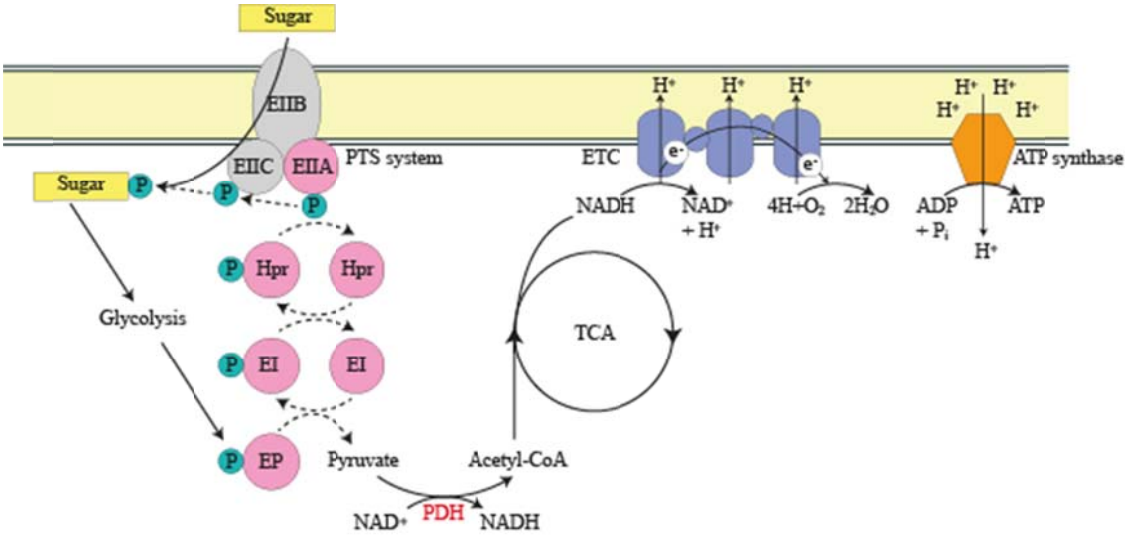

Figure 1. Schematic representation of central steps of carbohydrate uptake and metabolism, including the PTS system, TCA, and oxidative phosphorylation with electron transport chain (ETC) and ATP synthase. For a detailed description of the model, please refer to the text. PTS part of figure modified from Görke and Stülke (2008).

CRP (reviewed by Görke and Stülke, 2008) is a global regulator important for CCR by affecting the expression of several genes (Babu et al., 2003; Gosset et al., 2004; Martínez-Antonio and Collado-Vides, 2003; Zheng et al., 2004; Zhang et al., 2005). CRP is the primary sensor of carbon availability in bacteria (Saier et al., 1995) and is activated through binding of cAMP. The cAMP-CRP complex activates or represses genes involved in the uptake and utilization of secondary carbon sources. Thus, when glucose is not available as a carbon source, phosphorylation of $EIIA^{Glc}$ leads to conversion of ATP to cAMP. The elevated concentration of cAMP activates CRP, resulting in an upregulation of genes involved in the consumption of secondary carbon sources. Zhang et al. (2005) suggested a 22 bp consensus CRP binding site based on 41 known CRP binding sequences to be 5´-AATTGTGATCTAGATCACATTT-3´. In addition to CRP, other transcriptional regulators, such as Mlc and Cra (formerly FruR), are involved in the regulation of PTS in *E. coli* (Deutscher et al., 2006).

Post-transcriptional regulations also play a role in the regulation of sugar uptake. One of the many targets of cAMP-CRP in *E. coli* is the repression of the *spf*-encoded Spot 42 sRNA (Polayes et al., 1988). This unstable 109 nt sRNA was found to accumulate in the cell during growth in the presence of glucose (Sahagan and Dahlberg,

1979), and overexpression of Spot 42 has been shown to cause reduced growth on succinate and to introduce a lag phase following the transfer from minimal to rich media (Rice and Dahlberg, 1982). Spot 42 has been shown to cause Hfq-facilitated binding to the *galETKM* mRNA, which leads to discoordinated expression of the operon (Møller et al., 2002a, 2002b), repressing genes involved in uptake of galactose during glucose growth. Recently, a microarray and reporter fusion study from Beisel and Storz (2011) reported that Spot 42 regulates at least 14 operons, most of them repressed, dominated by genes involved in uptake and catabolism of non-preferred carbon sources. Several of the operons were regulated at the transcriptional level by cAMP-CRP as well, suggesting Spot 42 and CRP to control gene regulation through feed-forward loops.

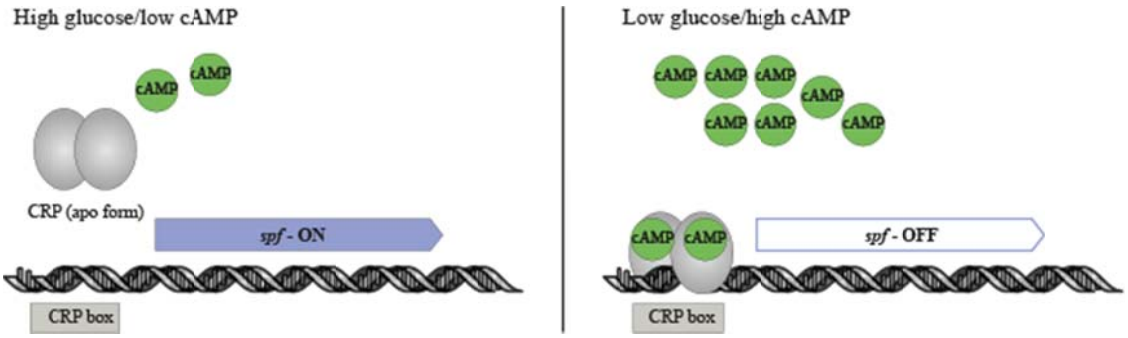

Figure 2. A model which describes CRP-dependent repression of *spf* (encoding Spot 42). Under conditions of high glucose/low cAMP, CRP is not activated, which leads to expression of *spf*. At low glucose/high cAMP, CRP is activated and functions as a repressor of *spf* expression.

Another sRNA involved in the regulation of PTS is SgrS (sugar transport-related sRNA) (Vanderpool and Gottesman, 2004). Accumulation of phosphorylated glucose in *E. coli* induces a stress response leading to inhibition of growth. SgrS is induced by the SgrR TF in response to intracellular accumulation of glucose-6-phosphate or the glucose analog α-methyl-glucoside-6-phosphate (Vanderpool and Gottesman, 2004). When glucose levels are high, SgrS negatively regulates the translation and stability of the *ptsG* mRNA, which encodes $EIIBC^{Glc}$. SgrS binds *ptsG* mRNA through a base-pairing mechanism facilitated by the RNA chaperone Hfq (Zhang et al., 2003). In addition to Spot 42 and SgrS, at least two other sRNAs, GlmY and GlmZ, and one *cis*-encoded

ribozyme play part in post-transcriptional regulation of sugar uptake and metabolism (reviewed by Görke and Vogel, 2008).

Bacteria use several systems for sugar transport into the cell. In addition to the major PTS system, ATP-driven systems involving periplasmic binding proteins (Ames, 1986; Davidson and Nikaido; 1991; Richarme et al., 1993) and ion-coupled symport systems using coupling ions as H^+ and Na^+ are in use (Wright et al., 1986; Kaback, 1986; Tsuchiya et al., 1977; Tsuchiya and Wilson, 1978; West, 1970). Facilitated diffusion of glucose in *Zymomonas mobiis* (Dimarco and Romano, 1985) and *Streptococcus bovis* (Russell, 1990) has also been reported. Halophilic ("salt loving") bacteria live in a Na^+ rich environment and utilize the high Na^+ concentration for transport of various nutrients. In *Vibrio alginolyticus*, most amino acid transport systems (Tokuda et al., 1982; Tokuda and Unemoto, 1982) and the sucrose transport system (Kakinuma and Unemoto, 1985) are dependent on Na^+. *Vibrio parahaemolyticus* has been shown to possess a PTS system for the transport of glucose into the cell (Kubota et al., 1979), but a Na^+/glucose symport system is present as well (Sarker et al., 1994).

Inside the cell, the carbohydrates undergo catabolism releasing its energy as high-energy compounds like ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Carbohydrate breakdown is initiated by glycolysis, a central metabolic pathway that appeared early in the evolution of life, which splits glucose into pyruvate with subsequent release of energy (Mathews et al., 1999; Canback et al., 2002). The 10 reactions are highly conserved across almost all living organisms and can proceed during both anaerobic and aerobic conditions (Mathews et al., 1999; Commichau et al., 2009). During aerobic conditions, pyruvate is usually prone to cellular respiration and is oxidized to acetyl-CoA and carbondioxide by the pyruvate dehydrogenase complex (PDH) (Patel and Roche, 1990). This reaction is also known as the *link reaction* as it links glycolysis to the tricarboxylic acid cycle (TCA). Acetyl-CoA enters TCA in reaction with oxaloacetic acid forming citric acid. Through an eight-step process involving 18 enzymes, the cycle ends up with four-carbon oxaloacetic acid, ready to react with acetyl-CoA to start a new cycle (Mathews et al., 1999). Following TCA, the electron transport chain (ETC) and oxidative phosphorylation take place at the bacterial cell membrane. ETC establishes a proton gradient across the inner membrane through

oxidizations of NADH (Mitchell and Moyle, 1967). ATP synthase utilizes the proton gradient to drive the phosphorylation of ADP to ATP, using exogenous oxygen as the final electron acceptor.

Glycolysis-formed pyruvate can also be oxidized using an endogenous electron acceptor in a process known as fermentation. This usually occurs during anaerobic conditions, but some microorganisms prefer fermentation to oxidative phosphorylation in aerobic conditions with readily available sugars (Kruckeberg and Dickinson, 1999). Under anaerobic conditions, fermentation is important to maintain the production of ATP through glycolysis. Pyruvate is oxidized to waste products like lactic acid or ethanol, leaving the electron carriers available for another round to glycolysis. This partly oxidation of pyruvate is less efficient than oxidative phosphorylation (Mathews et al., 1999). Microorganisms, primarily prokaryots living in an environment without oxygen, can also go through respiration using an inorganic electron acceptor in a process known as anaerobic respiration (Lovley and Coates, 2000). This process still uses ETC, but instead of oxygen, it utilizes exogenous inorganic compounds like sulphate, nitrate, or sulphur as the final electron acceptor.

1.3.2. The Fur regulon and iron homeostasis

Iron is required by almost all life forms and plays an important role in diverse biological processes, such as oxygen transport, electron transfer, and DNA synthesis. The iron is often found as incorporated into proteins or as iron-sulphur clusters or heme groups. Iron has a wide range of oxidation-reduction potentials and exists under physiological conditions at two stable valences, +2 and +3. In neutral or alkaline environments, iron is poorly soluble, which makes the concentration of iron in vertebrate hosts too low for normal bacterial growth to occur (Schaible and Kaufmann, 2004). The eukaryotic hosts keep iron solubilised in proteins with high affinity for iron in the serum, lactoferrin stored in the macrophages, or deposited at sites of infection. Inside a eukaryotic host, pathogenic bacteria can use highly developed iron sequestering systems to excrete siderophores with high affinity and specificity for Fe^{3+} to "steal" iron from the host (Griffiths, 1991; Collins, 2003). Such systems are important for virulence in several fish pathogenic bacteria, including *Vibrio anguillarum* (Crosa, 1980), *Vibrio vulnificus* (Amaro et al.,

1994), *Aliivibrio salmonicida* (Colquhoun and Sørum, 2001), and *Photobacterium damselae* ssp. *Piscicida* (Magarinos et al., 1994). As reported by McHugh et al. (2003), *E. coli*, in which iron metabolism is well understood, contains at least 35 genes encoding seven iron-acquisition systems, including four systems of which are siderophore based.

During aerobic conditions, excessive iron can convert hydrogen peroxide into hydroxyl radicals through the Fenton reaction, which makes free iron ions toxic to the bacteria. (Touati, 2000). To balance the uptake of iron (whenever it is available) to its cytotoxic properties, the cells contain highly developed iron-responsive regulatory systems. Regulation of iron homeostasis is in most studied bacterial species taken care of by the TF Fur (Hantke, 2001). At high intracellular levels of soluble iron, Fur normally binds Fe^{2+} and acts as a homodimer to negatively regulate genes involved in iron acquisition and iron storage by binding to a conserved TFBS, termed the Fur box. The Fur binding site is located inside the promoter region of genes part of the Fur regulon. The sequence 5´-GATAATGATAATCATTATC-3´ has been suggested as a consensus sequence for the *E. coli* Fur box (de Lorenzo et al., 1987; Griggs and Konisky, 1989). However, DNase I footprint studies show that the region protected by Fur is larger than the Fur box itself (Butterton et al., 2000; Watnick et al., 1998). The Fur binding region often consists of two or more overlapping or adjacent Fur boxes, which makes the Fur binding site extend up to 100 bp (Escolar et al., 2000), possibly by binding multiple Fur dimers wrapped around the double helix in a screw-like manner (de Lorenzo et al., 1988; Frechon and Le Cam, 1994). In addition to fur acting as the "classic" Fe^{+}-bound repressor, it has been shown to be involved in both positive and negative regulation in absence and presence of iron (for a review on Fur, see Carpenter et al., 2009).

During the last century, a number of studies have used microarray analyses of wild-type and *fur* deletion mutants in response to iron availability to identify genes that are part of the Fur regulon. These studies have been performed in various bacteria, such as *E. coli* (McHugh et al., 2003), *V. cholerae* (Mey et al., 2005a), *P. aeruginosa* (Ochsner et al., 2002), *Yersinia pestis* (Zhou et al., 2006), *Helicobacter pylori* (Ernst et al., 2005), *B. subtilis* (Baichoo et al., 2002), *Neisseria meningitides* (Grifantini et al., 2003), and *Campylobacter jejuni* (Holmes et al., 2005). In addition to genes directly involved in iron metabolism, these studies revealed Fur involvement in cellular processes as varied as

DNA metabolism, energy metabolism, redox-stress resistance, chemotaxis, metabolic pathways, bioluminescence, swarming, and production of toxins and other virulence factors, making Fur a true global regulator (Escolar et al., 1999; Hantke, 2001; McHugh et al., 2003; Wyckoff et al., 2007).

In addition to the important role of Fur as a repressor of iron acquisition genes at high intracellular levels of iron, a number of genes involved in iron storage, iron metabolism, and antioxidant defence seem to be positively regulated by Fur and iron (Niederhoffer et al., 1990; Park and Gunsalus, 1995; Quail et al., 1996; Tseng, 1997; Dubrac and Touati, 2000; Massé and Gottesman, 2002). Direct activation of gene expression by Fur has been reported (Delany et al., 2001; Delany et al., 2004), but most cases of positive regulation includes the involvement of the sRNA RyhB. RyhB has been reported to down-regulate at least 18 operons in *E. coli*. (Massé and Gottesman, 2002). The sRNA normally acts as a negative post-transcriptional regulator on a number of genes, and because *ryhB* itself is under control of a Fur box and is negatively regulated by Fur, the net outcome is positive regulation when iron is replete. However, RyhB itself has also been found to act as a positive regulator (Prévost et al., 2007).

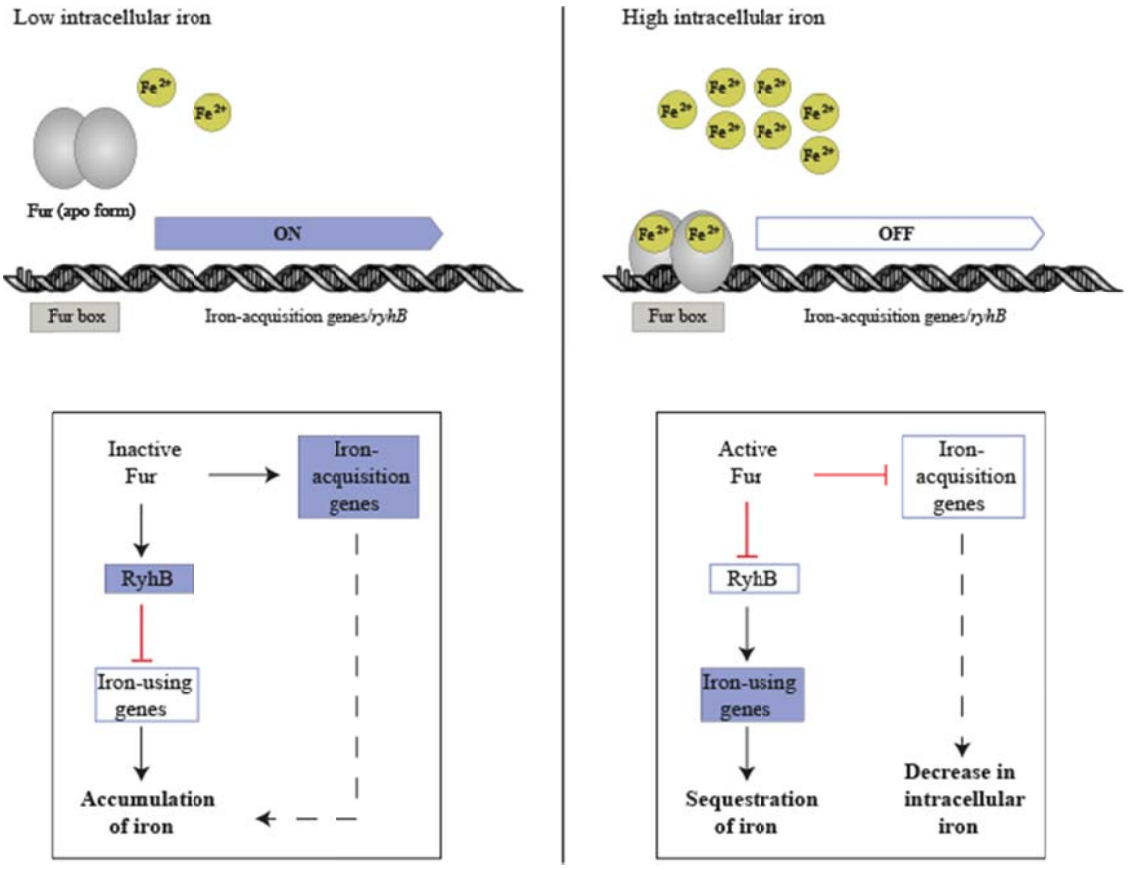

Figure 3. A model which describes Fur-dependent gene expression. Under conditions of low intracellular iron, Fur is not activated, leading to expression of iron-acquisition genes and *ryhB*. The net result is accumulation of iron. At high intracellular iron, Fur acts as a repressor on iron-using genes and *ryhB*. The net result is sequestration of iron and a decrease in the intracellular iron concentration. Figure modified from Massé and Arguin, 2005.

2. The *Vibrionaceae* family

The fish pathogen *A. salmonicida*, which belongs to the *Vibrionaceae* family (vibrios), has in this work been used as a model bacterium to study gene regulation. The *A. salmonicida* as a species and the *Vibrionaceae* family as a whole will therefore be described in further detail in this section of the thesis. *Vibrionaceae* consists of Gram-negative bacteria that belong to the gamma-subdivision of the *Proteobacteria* phylum (Garrity, 2004). With more than 115 recognized and74 validly described species (Silveira et al., 2010; Thompson et al., 2004), the vibrios have a broad host range susceptible to infection, including severe human pathogens causing diseases like cholera (*V. cholerae*),

gastroenteritis (*V. paraheamolyticus*), and septicemia (*V. vulnificus*) (Chakraborty et al., 1997). Vibrios are highly abundant in aquatic environments, including marine habitats, and are associated with a wide range of organisms. The non-pathogenic *Aliivibrio fischeri* and *Aliivibrio logei* may be found in symbiotic relationships with fish and squid (Haygood, 1993; Ruby and McFall-Ngai, 1999), whereas *V. anguillarum*, *A. salmonicida*, and *V. vulnificus* are significant fish pathogens, and *Vibrio harveyi* causes disease in shrimps (Thompson et al., 2004).

The vibrios have a curved rod appearance and are polarily flagellated and highly motile. They require NaCl for growth and have a facultative fermentative metabolism (Thompson et al., 2004). Their optimum growth temperature range from 7 to 40 ºC (Raguénès et al., 1997), thereby exposing them to psycrophilic and mesophilic growth conditions. Currently, there are 22 complete vibrio genomes in the NCBI Genomes database, including the model organism of our research group, *A. salmonicida*, whereas 65 and 24 have status as "Assembly" and "In progress", respectively. (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). All the complete genomes possess two circular chromosomes, one being larger than the other, with a total size ranging from 4-6 Mb. Some of the environmental strains, like *A. salmonicida, A. fischeri, V. harveyi*, and *V. vulnificus*, also contain one or more plasmids.

2.1. sRNA studies in *Vibrionaceae*

The majority of knowledge on sRNAs from *Vibrionaceae* originates from *V. cholera*. sRNAs have mainly been identified using computational predictions (mainly in IGRs), microarray experiments, shotgun cloning of cDNA libraries, cloning of abundant small RNAs, and co-purifications with proteins like Hfq, CsrA, and RNA polymerase (Huttenhofer and Vogel, 2006; Vogel and Sharma, 2005; Silveira et al., 2010). Experimentally verified vibrio sRNAs include the iron-regulated RyhB, shown in *V. cholerae* to be involved in regulation of multiple cellular processes like iron homeostasis, motility, chemotaxis, and biofilm formation (Davis et al., 2005; Mey et al., 2005a, 2005b), CsrB/CsrC/CsrD and CsrB1/CsrB2 in *V. cholerae* and *A. fischeri*, respectively, targeting the CsrA protein (Lenz et al., 2005; Kulkarni et al., 2006), Qrr regulating mRNAs involved in quorum sensing in *V. cholera*, *V. parahaemolyticus*, *V. vulnificus*, *V.*

harveyi, *A. fischeri*, and *V. anguillarum* (Lenz et al., 2004; Tu & Bassler, 2007; Miyashiro et al., 2010; Weber et al., 2008), MicX negatively regulating an outer membrane protein and the periplasmic component of a peptide ABC transporter in *V. cholera* (Davis and Waldor, 2007), VrrA modulating colonization and affecting release of outer membrane vesicles in *V. cholerae* (Song et al., 2008), TarA regulating the expression of *ptsG*, encoding a major glucose transporter, in *V. cholerae* (Richard et al., 2010), and MRB RNA I regulating plasmid replication in marine bacteria (Le Roux et al., 2010). In addition, characterization of a *V. cholera hfq* deletion mutant suggests that growth inside the intestine requires additional sRNAs (Ding et al., 2004). In 2005, a global computational sRNA search independent of TFBSs in *V. cholerae* identified nine out of 10 putative or known at that time *V. cholerae* sRNAs and 32 novel candidates, six of which were verified experimentally (Livny et al., 2005). Another study in *V. cholera* using direct cloning, depletion of 5S/tRNA, and parallel sequencing, identified all 20 known *V. cholerae* sRNAs, all of which were verified experimentally (Liu et al., 2009). In addition, 500 new putative sRNAs located in IGRs and 127 putative antisense sRNAs were predicted, of which seven and nine, respectively, were verified by Northern blot experiments. Preliminary functional characterization suggested one of the IGR-located sRNAs, IGR7, to be involved in carbon metabolism. Recently, a computational study exploring the ncRNA diversity in four sequenced environmental vibrio species, *V. alginolyticus* 40B, *Vibrio communis* 1DA3, *Vibrio mimicus* VM573, and *Vibrio campbellii* BAA-1116, resulted in 31-38 putative ncRNAs per specie (Silveira et al., 2010). Of the 38 predicted ncRNAs in *V. campbellii*, 21 were experimentally validated by microarray analyses.

2.2. Studies on Fur regulons in *Vibrionaceae*

While specific genes and parts of the Fur regulon have been the focus of various studies in many members of the *Vibrionaceae* family, whole-genome studies of the iron and Fur regulon have mainly been performed in *V. cholerae*. In a microarray study by Mey et al. (2005a), gene expression in wild-type strain O395 grown in presence or absence of iron was compared with a *fur* deletion mutant. The study identified 65 genes that were repressed under low iron conditions and in the *fur* deletion mutant, 14 of which were

novel and negatively regulated by iron and Fur. The identified genes encode products with function in vibriobactin synthesis, siderophore transport, heme transport, TonB systems, iron storage, regulatory proteins, stress response, and several of unknown function. The study also identified genes involved in other regulatory patterns, including positive regulation of gene expression by iron and Fur, regulation by iron independent of Fur, and vice versa. Several of the identified genes were also predicted to have a potential Fur box located in their promoter region.

In contrast to *E. coli* where Fur is negatively auto-regulated under iron-replete conditions (deLorenzo et al., 1988), a Fur box has not been identified in the promoter region of several vibrios (Litwin et al., 1992; Yamamoto et al., 1997; Colquhoun and Sørum, 2001; Mey et al., 2005a). However, the level of Fur mRNA increases two folds in iron-replete conditions (Lee et al., 2003), possibly by positive regulation by Fur binding to an AT-rich sequence (Lee et al., 2007).

2.3. *A. salmonicida* and cold water vibriosis

The Gram-negative *A. salmonicida* is shaped as a curved rod and contains up to 10 polar flagella (Holm et al., 1985). These features make the bacteria highly motile. Moreover, *A. salmonicida* is psycrophilic with an optimum growth temperature ranging from 10-15 ºC (Colquhoun et al., 2002) and moderate halophilic requiring 0.5-4.0 percent NaCl for growth (Egedius et al., 1986). *A. salmonicida* is the disease causing agent of cold-water vibriosis (aka. Hitra disease or hemorraghic syndrome) in Atlantic salmon (*Salmo salar* L.), captive Atlantic cod (*Gadus morhua* L.), and farmed rainbow trout (*Salmo Gairdneri* R.) (Egedius et al., 1981; Egedius et al., 1986; Jørgensen et al., 1989). The disease is observed in most North Atlantic countries part of the fish farming industry, such as Scotland (Bruno et al., 1985), Iceland and the Faroe isles (Dahlsgaard et al., 1988), and Canada and USA (O'Halloran et al., 1993; Griffiths and Salonius, 1995).

Cold-water vibriosis is, as its name implies, normally seen at low water temperatures (i.e., below 10 ºC; Enger et al., 1991), and upon infection, the fish experiences tissue degradation, hemolysis, and sepsis. The disease is currently under control by a vaccination program using formalin fixated whole bacterial cells (Holm and Jørgensen, 1987; Lillehaug, 1990), but information regarding the molecular mechanisms

of the different stages of the disease is still limited. However, observed pathology of infected fish suggests *A. salmonicida* to secrete exotoxins and cytolytic enzymes, as seen in other fish pathogens (Toranzo and Barja, 1993). Colquhoun and Sørum (2001) reported that siderophore production was only observed at temperatures below 10 ºC, suggesting the iron acquisition systems to play a role in *A. salmonicida* virulence. The same study also identified three high-molecular weight outer membrane proteins that were up-regulated under iron-restricted growth at low temperatures (6 and 10 ºC) but not at higher temperatures (15 ºC). Moreover, the production of hydrogen peroxide has been suggested as a possible virulence factor in *A. salmonicida* (Fidopiastis et al., 1999), and fish skin mucus supplemented growth showed an upregulation of flagellar proteins and proteins involved in oxidative stress responses (Raeder et al., 2007). As reported in Hjerde et al. (2008), several protein secretion systems and two haemolysins might play central roles in the virulence of *A. salmonicida*. The genomic analyses also revealed three *tonB* systems and one heme uptake system which are usually found to play part in iron acquisition.

2.4. *A. salmonicida* as a model bacterium for gene regulation studies

The lack of information on the mechanisms of cold-water vibriosis inspired us to use *A. salmonicida* as a model to study global gene regulation and perhaps use the results as basis to better understand the disease. The complete genome sequence of *A. salmonicida* strain LFI1238 was recently published (Hjerde et al., 2008). It revealed that the genome follows the consensus from the other *Vibrionaceae* members and harbours two chromosomes, one larger than the other (3.3 and 1.2 Mb), and four plasmids of 83.5, 30.8, 5.4, and 4.3 kb. Phylogenetic studies of 16S rDNA have suggested *A. salmonicida* to be closest related to *A. fischeri* and *A. logei* (Wiik et al., 1995; Fidopiastis et al., 1998), which are both known for their luminous behaviour. When comparing all the protein coding sequences of *A. salmonicida* to those of the other published genomes of the *Vibrionaceae* familiy, the highest number of orthologous genes was shared with *A. fischeri* (70 percent), whereas the other *Vibrionaceae* members had on average 55-60 percent shared orthologs (Hjerde et al., 2008).

II. Aims of the study

Main objective

The main objective of this study was to use genome-wide prediction and transcriptome approaches to achieve a better understanding of gene expression regulation in the Gram-negative fish pathogen *A. salmonicida*.

Secondary objectives

(1) Identify all Fur-regulated genes and operons in vibrios and increase the knowledge about the Fur regulon by genome-wide predictions.

(2) Gain further insights into of post-transcriptional regulation by sRNAs on a genome-wide scale, using both computer-based and experimental-based methods.

(3) Generate detailed knowledge on selected sRNAs.

III. Summary of papers

Paper 1

Rafi Ahmad, Erik Hjerde, Geir Åsmund Hansen, Peik Haugen, Nils-Peder Willassen (2008). **Prediction and Experimental Testing of Ferric Uptake Regulator Regulons in Vibrios.** *J Mol Microbiol Biotechnol*. 16:159-68.

The intracellular levels of iron are carefully regulated in bacteria and are mediated by the ferric uptake regulator (Fur) in many bacterial species. During conditions of high intracellular iron, Fur represses expression of genes involved in iron acquisition and iron storage through binding of a conserved sequence motif, termed the Fur box, located within the promoter region. Knowledge into unique members of the Fur regulon in vibrios can potentially provide insights into virulence and pathogenesis. In this study, we have constructed a vibrio-specific alignment matrix based on Fur binding sites from the literature, which resulted in a Fur box consensus sequence of 5′-AATGANAATNATTNTCATT-3′. Five published vibrio genomes and the draft genome of *Vibrio salmonicida* were included in a Fur box prediction using Patser software. Fur binding motifs were identified in front of 50–61 single genes and 16–20 operons in each genome. In addition, eight single genes and four operons previously not described as regulated by Fur where predicted in all six vibrio genomes. Interestingly, a plasmid-encoded iron ABC-transporter operon unique to *Aliivibrio salmonicida* was identified. A subset of the predicted Fur-regulated genes was experimentally tested using Northern blot analysis. Expression data from six previously not described Fur-regulated genes showed increased mRNA levels during iron-restrictive conditions. The results also suggested a correlation between the iron responsiveness of genes with the number of Fur boxes and their Patser score.

Paper 2

Rafi Ahmad, Geir Åsmund Hansen, Hilde Hansen, Erik Hjerde, May Liss Julianne Nyrud, Nils-Peder Willassen, Peik Haugen (2010). **Prediction, microarray, and Northern blot analyses identify new intergenic small RNAs in *Aliivibrio salmonicida*.** *PLoS ONE*. Under revision.

Bacterial small RNAs (sRNAs) are typically transcribed in *trans* from separate promoters in the intergenic regions and act by binding mRNA by short sequence complementarities, thereby changing the expression of the corresponding protein. Some 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, there are still many sRNAs to be found, and the work of identifying functional roles of most sRNAs has just started. For example, in *Vibrionaceae*, many hundreds candidate sRNAs have been predicted, but a functional role is only known for nine. In this work, we have used computational and experimental methods to identify intergenic non-coding RNAs (mostly sRNAs) in the marine bacterium *Aliivibrio salmonicida*. A computational search for non-coding RNAs limited to the intergenic regions of the 4.6 Mb genome identified a total of 252 potential ncRNAs, 233 of which were putative sRNAs. In total, we identified 50-80 putative ncRNAs, depending on the set threshold value for fluorescence signal in our microarray approach. Twelve of these, including nine novel sRNAs, were verified by Northern blot analysis.

Paper 3

Geir Åsmund Hansen, Rafi Ahmad, Erik Hjerde, Christopher G. Fenton, Nils-Peder Willassen, Peik Haugen (2011). **Expression profiling of a *spf* deletion mutant suggests biological roles and mRNA targets for Spot 42 in the fish pathogen *Aliivibrio salmonicida*.** Submitted to *RNA Biology*.

The small regulatory RNA (sRNA) Spot 42, encoded by the *spf* gene, was identified in *Escherichia coli* nearly 40 years ago. Its function and targets remained obscure until it was shown to cause discoordinate expression of the galactose operon. Recently, Spot 42 has also been reported to be involved in the regulation of the central and secondary metabolism. The *spf* gene is ubiquitous in the *Vibrionaceae* family of gamma-proteobacteria. A member of this family, the fish pathogen *Aliivibrio salmonicida*, encodes a Spot 42 homolog with 84 percent identity to *E. coli* Spot 42 (*spf*). In this study, we have generated a *A. salmonicida spf* deletion mutant and used trancriptome analyses to provide insights into the biological roles of Spot 42 in this bacterium, using microarray and Northern blot analyses to monitor expression. During conditions of glucose growth, a surprisingly large number of genes were ≥2 folds up-regulated and several major cellular processes were affected, such as carbohydrate metabolism and transport, motility and chemotaxis, iron homeostasis, and quorum sensing. Interestingly, a gene encoding a pirin-like protein (VSAL_I1200) responded to presence/absence of glucose in an on/off expression pattern, suggesting Spot 42 to regulate the important switch between fermentation and respiration in central metabolism. In addition, in a global search we identified another sRNA, named VSsrna24, encoded immediately downstream in the same intergenic region as *spf*. Expression of *VSsrna24* is highly dependent on glucose, and it is expressed in an opposite expression pattern compared Spot 42. Our hypothesis is that this novel sRNA works in concert with Spot 42 to regulate carbohydrate metabolism and uptake.

Paper 4

Geir Åsmund Hansen, Rafi Ahmad, Nils-Peder Willassen, Peik Haugen (2011). ***Aliivibrio salmonicida*** **encodes a small RNA immediately downstream of *spf*: Microarray analysis and potential roles in carbohydrate transport and metabolism.** Manuscript (for submission to *PLoS ONE*).

Bacterial small regulatory RNAs (sRNAs) are involved in the regulation of important cellular processes, such as stress responses, metabolism, quorum sensing, motility, and more. In addition, sRNA regulation plays key roles in virulence, for example in the gene regulation associated with formation of biofilms. With a lot of accumulating sRNA data, the work of mapping their biological roles in bacteria has just started. In this study, we have searched for potential biological roles of the recently reported sRNA in *Aliivibrio salmonicida*, VSsrna24. We have construction a *VSsrna24* deletion mutant and monitored transcriptome changes using microarray and Northern blot analyses. In presence of glucose, a large number of genes were differentially expressed (≥2 folds), including genes involved in the regulation of cellular processes such as carbohydrate metabolism and uptake, motility, and chemotaxis. Two NagE homologs, VSAL_I0831 and VSAL_II0721, were up-regulated in the *VSsrna24* deletion mutant. We hypothesise that these genes are involved in the uptake of glucose into the cell. Interestingly, several up-regulated genes in the VSsrna24 deletion mutant correspond to up-regulated genes in the previously reported Spot 42 knock-out mutant. In addition, several genes involved in motility and chemotaxis were found to be down-regulated in the *VSsrna24* deletion mutant. This is opposite of what was reported in the Spot 42 knock-out mutant where a large number of genes from the same category were up-regulated.

IV. Discussion

This project started out with a computational genome-wide prediction of sRNAs and Fur binding sites, followed by a subsequent experimental validation of these predictions. Next, more detailed experimental studies were performed using gene deletion technology and transcriptome analyses on two selected sRNAs, i.e., the Spot 42 homolog and the novel VSsrna24 in *A. salmonicida*. This part of the thesis will include some of the main findings from the papers and a general discussion on three selected topics. First, the use of sRNAs instead of transcriptional regulators in regulatory networks will be discussed. The studied regulatory networks of iron homeostasis and uptake of carbohydrates will serve as examples. Second, prediction of TFBSs and the Fur box in particular will be discussed. This section will also include a discussion on sRNA predictions and the importance of antisense RNAs. Finally, potential co-regulation of genes by Spot 42 and VSsrna24 will be discussed in detail.

1. Regulations by sRNAs compared to transcriptional regulators

Bacteria have evolved to include sRNAs as part of their regulatory networks (Beisel and Storz, 2010). Even though the signal transduction in most cases could have been achieved by other regulators, including transcription factors, regulations at the RNA level play an important role. It has been suggested that these conserved regulatory RNAs are relics from the "RNA world" (Gilbert, 1986; Jeffares, 1998), and that at least one sRNA plays part in the regulation of each major adaptive stress responses (Gottesman, 2004; Backofen and Hess, 2010). However, regulations through sRNAs provide some unique advantages over protein regulators. For example, the sRNAs are small in size and are not translated, giving their action a very short response time and potentially a low metabolic cost (Mizuno et al., 1984; Altuvia et al., 1997; Massé and Gottesman 2002; Beisel and Storz, 2010). Also, the sRNAs act post-transcriptionally and work independent of and epistatic to any transcriptional signals for their target mRNAs (Gottesman, 2004; Gottesman, 2005). Furthermore, one sRNA can simultaneously regulate many genes independent of the inducing signals for each of the genes, and multiple sRNAs, each transcribed under different conditions, might regulate a single target under different cellular conditions (Gottesmann, 2004).

1.1. The CRP/Spot 42 regulatory network

Results from **Paper 3** identify players in a regulatory network in *A. salmonicida* that involve both a transcriptional regulator and an sRNA, i.e., regulations by CRP and Spot 42. In *E. coli*, the expression of *spf*, the gene encoding Spot 42, is negatively regulated by a cAMP-CRP complex, which binds to a CRP box in the promoter region (Møller et al., 2002a). A CRP box is also identified upstream of the *A. salmonicida* Spot 42 homolog, and expression of the sRNA is responding to cAMP and glucose in a similar manner as in *E. coli*, suggesting *A. salmonicida* CRP to regulate Spot 42 similar as in *E. coli*. In Beisel and Storz (2011), *E. coli* Spot 42 was reported to participate in a feed-forward loop together with CRP, thereby down-regulating genes involved in the uptake of non-preferred sugars. To play part in a feed-forward loop, both regulators, in this case CRP and Spot 42, must regulate the same gene, and one of the regulators must regulate the other, such as CRP regulation of Spot 42 expression.

In *A. salmonicida*, six, eight, one, and three genes involved in PTS, anaerobic respiration, glycolysis, and electron transport (cytochromes and redox), respectively, were significantly (≥2 folds) up-regulated in the *spf* deletion mutant (**Paper 3**). At least two of these PTS genes [i.e., two gluconate permease homologs (VSAL_I2593 and VSAL_II0665)] have a potential CRP box in their promoter region. VSAL_I2593 and VSAL_II0665 have possible CRP boxes of 5′-CACTTTGTGCATCTACACAATA-3′ and 5′-TAATCTGAGCGAGATCTCATTT-3′ that are located 123 and 143 bp, respectively, upstream of predicted translation start. These CRP boxes are differing 12 and six bp, respectively, compared to the 22 bp CRP consensus sequence of 5′-AATTGTGATCTAGATCACATTT-3′ (Zhang et al., 2005). Two CRP boxes are also identified in front of the *E. coli* gluconate permease *gntP* (Zheng et al., 2004). Given that the genes in *A. salmonicida* are also regulated by CRP, then they could potentially be regulated by a feed-forward loop mechanism together with Spot 42. Feed-forward loops with CRP activating target genes and Spot 42 repressing the same genes have been shown to reduce leaky expression during repressing conditions, i.e., in presence of glucose (Beisel and Storz, 2011). The same study also reported that Spot 42 caused a delay in target protein levels when CRP is activated, i.e., in the absence of glucose, and a faster

decrease when CRP is inactivated, i.e., during growth with added glucose. This ability has not been associated with feed-forward loops comprised of only transcription factors, suggesting a potential advantage of sRNAs over proteins (Beisel and Stroz, 2010).

In summary, a potential role of Spot 42 in *A. salmonicida* is to reduce leaky expression of uptake systems for other carbon sources than glucose during glucose growth, possibly in a feed-forward loop together with CRP. In a biological context, this makes sense as Spot 42 down-regulates the uptake systems for gluconate during glucose growth and allows the bacteria to adapt more rapidly when introduced to glucose.

1.2. The Fur/RyhB regulatory network

In **Paper 1,** another regulatory network which includes both a protein transcriptional regulator and an sRNA was studied in detail, i.e., the Fur/RyhB regulatory network. As reported in the study, Fur boxes were predicted in front of 60 single genes and 20 operons, of which eight genes/operons were validated experimentally. In addition, an AT-rich Fur binding site was predicted in front of *fur*, possibly involved in positive self-regulation of the gene. One of the validated Fur targets was the sRNA RyhB. In *E. coli*, the TF Fur represses expression of the sRNA RyhB when intracellular levels of iron are high. In contrast, when iron is scarce, Fur cannot repress expression of RyhB, and the sRNA down-regulates operons encoding iron-using proteins involved in various cellular processes, such as TCA, dismutation of superoxide radicals, and iron storage (Massé and Gottesman, 2002). The reason for having an sRNA as part of this regulatory network could potentially be because RyhB is short in length and requires no translation, thereby having the ability to react very fast to changes in the concentration of intracellular iron. Another reason is the ability of RyhB to turn Fur into an indirect activator (Beisel and Storz, 2010). The genes within the Fur regulon are generally repressed at high intracellular iron concentrations, but this does not apply to the genes down-regulated by RyhB, thereby switching Fur from a repressor into an indirect activator.

Fur and RyhB have also been suggested to regulate genes by feed-forward loops (Beisel and Storz, 2011). The *sdh* operon (making up the succinate dehydrogenase complex) is reported to be directly regulated by RyhB in *E. coli*, and the operon contains a predicted Fur binding site (Massé and Gottesman, 2002; Zhang et al., 2005). However,

the Fur box prediction in **Paper 1** did not identify any Fur binding sites in front of the *sdh* operon in *A. salmonicida*.

2. Prediction of TFBSs and sRNAs

With the constantly growing amount of available genomic data, especially complete genome sequences, comparative genomics play an important role in the work of mapping genomic features, such as promoters and TFBSs. Transcription of both protein coding genes and sRNAs are dependent on promoters (e.g., sigma factors) and/or TFBSs (e.g., Fur boxes) located in their promoter region. Prediction of such elements in bacteria is a challenging task that usually requires species-specific consensus sequences. Unfortunately, only a few promoters and TFBSs are experimentally validated in bacterial species other than *E. coli*.

2.1. Determination of a Fur box consensus sequence

Although Fur regulons have been thoroughly studied in several organisms, the consensus sequence of the Fur box and determination of which residues that are directly involved in the Fur-DNA binding have not been completely clarified. In **Paper 1**, a vibrio Fur box consensus sequence was generated. It was based on 66 experimentally verified or proposed Fur binding sites of known Fur-regulated genes. The Fur boxes were aligned manually and automatically, and both methods resulted in an identical 19 nt palindromic consensus sequence (i.e., 5´-AATGANAATNATTNTCATT-3´).

The Fur box consensus sequence from **Paper 1** is offset by three nucleotides when compared to the classic *E. coli* consensus [5´-GATAATGAT(A/T)ATCATTATC-3´ (de Lorenzo et al., 1987, 1988)] and nearly identical to the consensus in *B. subtilis* [5´-TGATAATTATTATCA-3´ (Fuangthong and Helmann, 2003)] and *Y. pestis* [5´-AATGATAATNATTATCATT-3´ (Zhou et al., 2006)]. Recently, the predicted vibrio and *Y. pestis* Fur box consensus have been experimentally verified to bind Fur *in vitro* (Pedersen et al., 2010; Gao et al., 2008).

Several models that explain how Fur binds to DNA have been suggested. The classic model was suggested to bind a single Fur dimer by a 19 bp inverted repeat centered on an A or T (de Lorenzo et al., 1987). The classic 19 bp consensus has also

been proposed as the binding site of overlapping 6-1-6 or 7-1-7 arrangements suggested to interact from opposite sides of the DNA helix (Baichoo and Helmann, 2002; Lavrrar et al., 2002; Lavrrar and McIntosh, 2003). Chen et al. (2007) suggested the minimal recognition unit of Fur to be at least 20 bp in length and proposed the M9 (9-1-9) model. Moreover, Escolar et al. (1998; 1999) proposed a six bp (5´-NAT(A/T)AT-3´) multiple repeat model to explain the apparent polymerization of Fur on DNA.

Recent publications on Fur binding sites from *Y. pestis* (Gao et al., 2008), *Acidithiobacillus ferrooxidans* (Quatrini et al., 2007), *N. meningitidis* (Grifantini et al., 2003), *E. coli*, *B. subtilis*, and *P. aeruginosa* (Chen et al., 2007) suggest alternative models compared to the classical *E. coli* Fur box. In addition, a study on Fur binding sites in *V. vulnificus* found Fur to bind a 37 bp sequence where the central 15 bp resembles the central part of the vibrio consensus. All these alternative models are in agreement with data from **Paper 1** that suggests Fur to bind a core 7-1-7 inverted repeat of 5´-TGATAATNATTATCA-3´ (or similar) centered around a degenerate nucleotide (N) positioned three nucleotides downstream relative to the center A/T of the classic Fur box (Figure 4). Altogether, data from **Paper 1** along with recent publications indicate a general Fur box that includes a core 7-1-7 inverted repeat with a consensus sequence similar to that from vibrios.

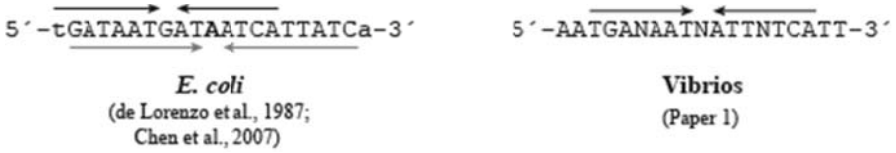

Figure 4. Fur box consensus sequence from *E. coli* and vibrios. The conserved 7-1-7 core inverted repeat of 5`-TGATAATNATTATAC-3` is shown by black arrows above the sequences. Nucleotides differing from this consensus are in bold. Gray arrows underneath *E. coli* sequence indicate the classic Fur box.

2.2. Computational prediction of sRNAs

Numerous potential sRNA genes have been predicted by studies using different computational methods (Backofen and Hess, 2010). However, because of inherent limitations, it has been suggested that these studies only have identified a subset of the total sRNA pool (Vogel et al., 2003; Kawano et al., 2005). Most bioinformatical

approaches are based on a combination of conserved features from known sRNAs, excluding sRNAs that do not fulfill the chosen criteria. A limitation in almost all of the computational approaches so far is restricting the search only to the IGRs of the genome. The majority of known sRNAs have been found in IGRs. However, by ignoring all other genomic regions, this approach excludes potential sRNA genes within protein coding regions. Moreover, predictions have been limited to a subset of genomic transcriptional elements and/or conservation in closely related species (Argaman et al., 2001; Wassarman et al., 2001). Rivas et al. (2001) were the first to include conservation of RNA secondary structure rather that primary sequence in their predictions. However, comparative studies are dependent on sRNAs being conserved among species, possibly missing out species-specific sRNAs.

The *A. salmonicida* whole genome sRNA prediction study performed in **Paper 2** utilized a combination of different strategies in the search for sRNA genes. The search was limited to the IGRs of the genome. Predictions of promoter recognition sites for sigma 70, 54, and 38 and the Fur TFBS identified in **Paper 1** were included. Sigma 70 binding sites are associated with "housekeeping" genes, sigma 54 controls motility, biofilm formation, luminescence, and colonization in *A. fischeri* (Wolfe et al., 2004), and sigma 38 is involved in transcription of genes associated with starvation and stationary phase. A search for Rho-independent terminators located ≥50 nt downstream of and in the same orientation as promoters/TFBSs was also included. The extracted IGRs from *A. salmonicida* were used to search for homologous IGRs in six other members of *Vibrionaceae* (*V. cholerae*, *A. fischeri*, *V. parahaemolyticus*, two *V. vulnificus* strains, and *Photobacterium profundum*) and subsequently to search for homologous putative sRNAs and conservation of gene synteny. To search for known homologs, the program cmsearch (Nawrocki et al., 2009) and Rfam database of non-coding RNA families (Gardner et al., 2009) were used.

Recently, a study by Liu et al. (2009) performed an unbiased experimental method exploring the pool of RNA by direct cloning and parallel sequencing, i.e., RNA-seq (aka. deep-sequencing). The study reported 500 new candidate sRNAs encoded in the IGRs and 127 candidate antisense sRNAs in *V. cholerae*. Of these, seven and nine, respectively, were validated experimentally through Northern blot analyses. In addition,

over one-third of the candidate sRNA reads corresponded to open reading frames, representing possible functional sRNAs, small peptides, or mRNA degradation products. With 64 percent of the total candidate sRNAs being encoded in the IGRs, representing only 12 percent of the *V. cholerae* genome, the RNA-seq results suggest that the IGRs are enriched for sRNAs over other regions of the genome.

The results from Liu et al. (2009) and RNA-seq studies in *L. monocytogenes* (Mraheil et al., 2011) and *Salmonella enterica* serovar Typhimurium (Sittka et al., 2008) also suggest that a significant number of candidate sRNAs genes are encoded antisense to, or within, protein coding genes. Yachie et al. (2006) combined predictions for ncRNA genes encoded in the IGRs with genes encoded antisense of coding regions in *E. coli*. The study identified 87 sRNA and 47 antisense RNA candidates, of which eight and four candidates, respectively, were experimentally validated. However, the used approach was less suitable for prediction of antisense RNAs compared to IGR sRNAs (Backofen and Hess, 2010). In a study by Georg et al. (2009), four different methods were used to identify antisense RNAs in cyanobacterium *Synechocystis* sp. PCC 6803. Of the total 73 identified antisense RNAs, 11 were computationally predicted based on identification of Rho-independent terminators.

To summarize, these studies suggest that the majority of sRNA genes are encoded in the IGRs of the genome. However, there are potentially a significant number of sRNA genes encoded antisense to, or within, coding regions to be discovered if searches are expanded to include protein coding regions. In addition, unbiased experimental methods such as RNA-seq are important to complement and validate existing sRNA prediction techniques.

3. *A. salmonicida* Spot 42 and VSsrna24 are involved in the same regulatory network

Rather than being constitutively expressed, the majority of sRNAs have been suggested to accumulate under different stress conditions (Vogel and Wagner, 2005). For example, the presence or absence of glucose in immediate surroundings of bacteria has the potential to trigger stress responses. In *E. coli*, accumulation of intracellular glucose-6-phosphate induces expression of the sRNA SgrS, which subsequently mediate

degradation of the glucose-specific PTS permease (PtsG) mRNA (Wadler and Vanderpool, 2007). In absence of glucose or other carbon sources, the bacteria may experience starvation stress responses. Genes expressed during starvation stress are often under control of the *rpoS*-encoded alternative sigma factor S. Interestingly, *rpoS* is itself regulated post-transcriptionally by at least four sRNAs, i.e., DsrA (Majdalani et al., 1998), RprA (Majdalani et al., 2002), ArcZ (Papenfort et al., 2009), and OxyS (Altuvia et al., 1997).

3.1. Spot 42 and VSsrna24 work in concert

Paper 3 and **Paper 4** identify hundreds of genes that are differentially expressed in *spf* and *VSsrna24* deletion mutants, respectively. Strikingly, the microarray studies reveal that a large number of the differential expressed genes are found in both datasets. For example, of the 19 and 29 differentially up-regulated genes in the Spot 42 and VSsrna24 mutants, respectively, that belong to the classes "Carbohydrates, organic acids and alcohols; PTS" and "Energy metabolism, carbon", 16 genes were found in both mutants. In addition, the microarray results and subsequent Northern blots suggested that the expression of *VSsrna24* was dependent on Spot 42, i.e., *VSsrna24* was not expressed in the *spf* deletion mutant. However, this could be caused by a change in the transcriptional regulation by knocking out Spot 42, and further studies are required to prove or disprove these assumptions.

The two studied sRNAs are located in the same IGR, and the expression of both sRNAs is responding to glucose. Moreover, expression of *spf*, but not *VSsrna24*, responds to cAMP. Expression studies reveal that *spf* is expressed in the presence of glucose, whereas *VSsrna24* is expressed in the absence of glucose. Therefore, we assume that Spot 42 plays a physiological role in presence of glucose, whereas VSsrna24 acts in conditions without glucose. The genomic location, expression patterns, and results from the microarray studies suggest that the two sRNAs play a role in the same regulatory networks under different conditions, working in concert with each other.

To further investigate potential co-regulation between Spot 42 and VSsrna24, the two sequences were bioinformatically aligned (figure 5). Of the 37 nt from VSsrna24 (excluding the terminator), 20 were identical to Spot 42 from both *A. salmonicida* and *E.*

coli. Compared to the potential RNA-binding single-stranded regions from *E. coli* Spot 42, four out of 10 and nine out of 18 nt were conserved in regions I and II, respectively, whereas region III was not covered by VSsrna24. This striking similarity between Spot 42 and VSsrna24 strengthens the hypothesis that the two sRNAs potentially work in concert and have similar roles.

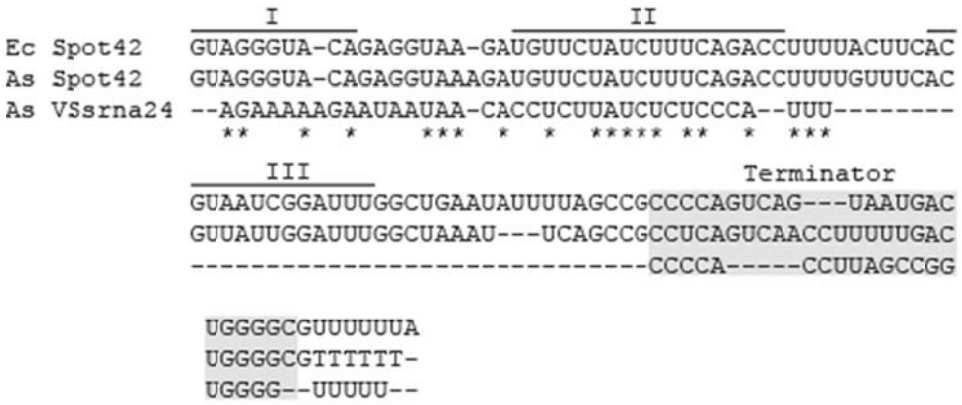

Figure 5. Alignment between Spot 42 from *E. coli* (Ec Spot 42) and Spot 42 and VSsrna24 from *A. salmonicida* (As Spot 42 and As VSsrna24). Nucleotides outside terminator identical to all three sequences are indicated by asterisks. Potential RNA-binding single-stranded regions from *E. coli* Spot 42 are labelled I, II and III (Møller et al., 2002a; Beisel and Storz, 2011).

Results from **Paper 3** and **Paper 4** suggest Spot 42 and VSsrna24 to play part in the regulation of genes involved in uptake and metabolism of carbohydrates. The possible co-regulation of the same genes by Spot 42 and VSsrna24 may be explained by the glucose dependent expression patterns of the two sRNAs. During glucose growth, Spot 42 possibly down-regulates uptake of other carbon sources than glucose in a CCR-like manner. Under non-glucose conditions, Spot 42 is no longer expressed, but expression of uptake systems for most carbon sources is still not required. Under such conditions, these systems might be down-regulated by the co-working VSsrna24.

Two genes possibly under control of both Spot 42 and VSsrna24 (i.e., significantly up-regulated in both deletion mutants) are VSAL_I2593 and VSAL_II0665, encoding two gluconate permeases. The *E. coli* homolog, *gntP*, encoding a gluconate permease involved in the uptake of D-fructuronate/D-gluconate (Klemm et al., 1996;

Bates Utz et al., 2004) has been suggested to be regulated by two sRNAs, OmrA and OmrB (Tjaden et al., 2006). In an attempt to identify potential common sRNA-mRNA binding, a computational binding prediction between the -100/+50 (relative to translation start) sequences of the two gluconate permeases and the conserved Spot 42 and VSsrna24 regions was performed using RNA hybrid (Krüger and Rehmsmeier, 2006). Figure 6A shows the binding potential between *VSAL_II0665* mRNA and Spot 42 and VSsrna24. Both sRNAs have the potential to bind *VSAL_II0665* mRNA mRNA in the same 26 nt area covering translation start. Based on the alignment in figure 5, six of the nucleotides involved in binding are identical in Spot 42 and VSsrna24. For *VSAL_I2593* mRNA, the area of binding potential for both Spot 42 and VSsrna24 is seven nt located 41 nt upstream of translation start (figure 6B). Three of these nucleotides are identical in Spot 42 and VSsrna24. The potential of Spot 42 and VSsrna24 to bind to same region in the two gluconate permeases suggests that the mRNAs might be regulated by both sRNAs.

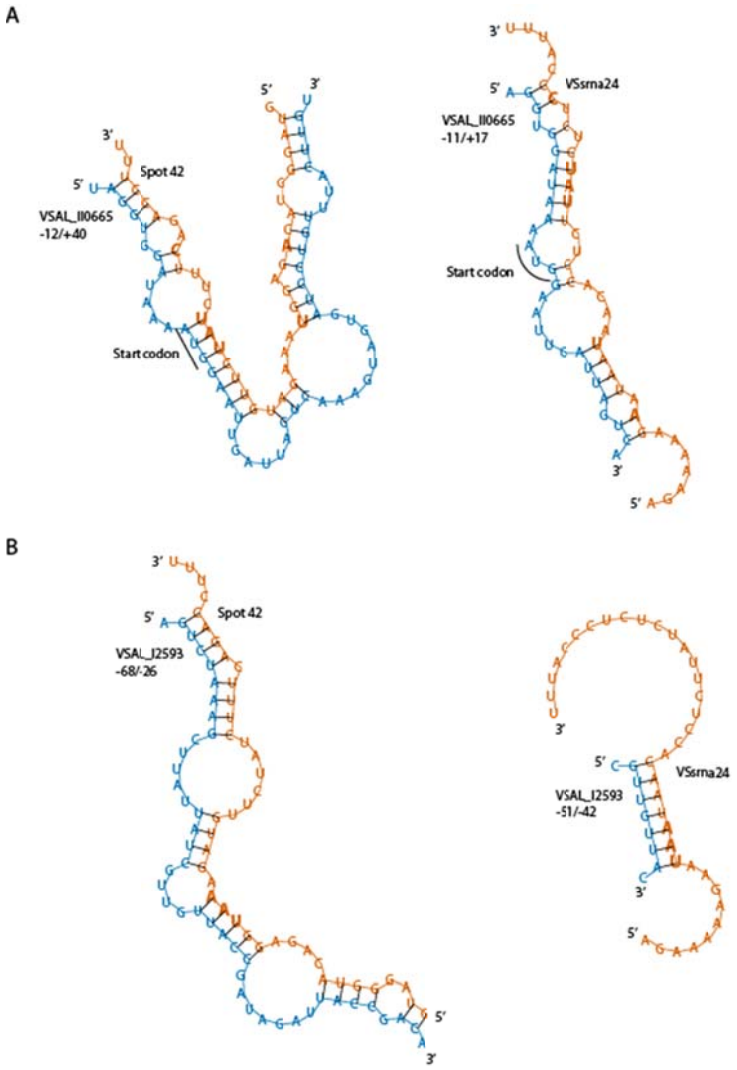

Figure 6. Binding potential between VSAL_II0665 and Spot 42 and Vssrna24 (A) and VSAL_I2593 and Spot 42 and Vssrna24 (B) generated using RNAHybrid (Krüger and Rehmsmeier, 2006). Nucleotides identical to both Spot 42 and VSsrna24 are in bold.

3.2. VSsrna24 down-regulates two PTS permeases, NagEI and EII, with possible affinity for glucose

In *E. coli* and *V. cholerae*, the major PTS glucose-specific permease EIIBC^{Glc} is negatively regulated by the sRNAs SgrS and TarA, respectively (Vanderpool and Gottesman, 2004; Richard et al., 2010). In *E. coli*, the expression of SgrS is activated by the regulator protein SgrR, which initiates downregulation of PtsG under conditions of phosphoglucose stress, thereby preventing further uptake of glucose into the cell. In. *V.*

cholera, the sRNA TarA, regulated by the major virulence gene activator ToxT, down-regulates PtsG during infection.

Interestingly, we have so far not been able to show expression of the *ptsG* homolog in *A. salmonicida*. In Northern blot experiments, two radio-labelled probes that cover different regions of the gene did not result in any bands, neither in presence or absence of glucose. However, the amino acid sequences of NagEI (VSAL_I0831) and NagEII (VSAL_II0721) in *A. salmonicida* have a global identity and similarity of 65 and 84 percent, respectively, and they both share a ~36 and ~66 percent identity and similarity to the PtsG amino acid sequence. In *E. coli*, the NagE N-acetylglucosamine PTS permease is homologous to PtsG/Crr, the glucose-specific PTS enzyme II (Peri and Waygood, 1988), and it has been proposed that the two transport and phosphorylation systems have evolved from a common ancestral gene (Rogers et al., 1988). Moreover, a PtsG homolog has not been found in *Vibrio furnissii*, but its NagE has shown the ability to translocate both glucose and N-acetylglucosamine (Bouma and Roseman, 1996). *A. salmonicida* NagEI and NagEII have 85 and 68 percent identity and 91 and 82 percent similarity with query coverage of 98 and 99 percent, respectively, to *V. furnissii* NagE. These results support that NagEI and NagEII are involved in the uptake of glucose in *A. salmonicida*. In addition, figure 7 shows that VSsrna24 has the potential to bind both *nagEI* and *nagEII* mRNAs close to translation start. The downregulation of NagEI/NagEII by Vssrna24 in *A. salmonicida* in absence of glucose suggests that the PTS uptake system for N-acetylglucosamine, and possibly glucose, is not expressed during such conditions. This assumption makes biological sense because the bacteria do not require uptake systems for glucose when it is not present in the surroundings.

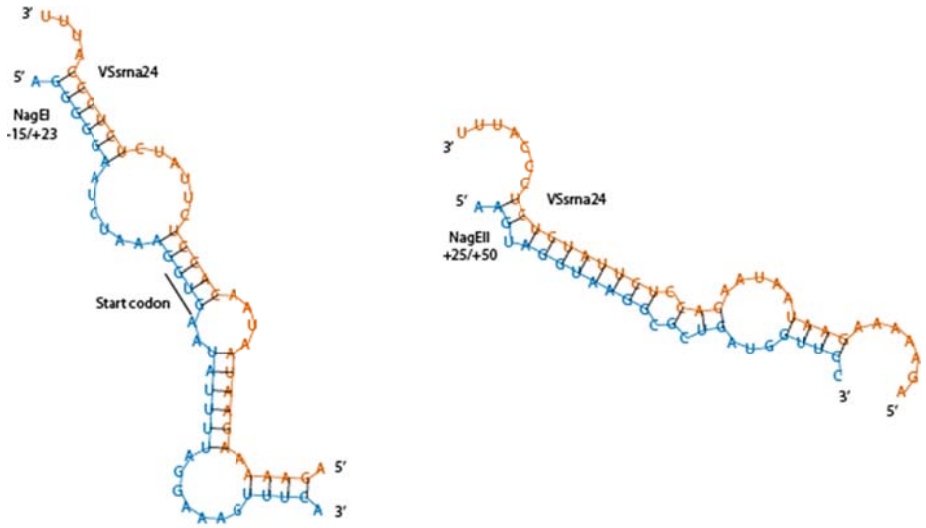

Figure 7. Binding potential between VSsrna24 and NagEI (VSAL_I0831) and NagEII (VSAL_II0721) generated using RNAHybrid (Krüger and Rehmsmeier, 2006).

3.3. Spot 42 and Vssrna24 modulate the expression of motility and chemotaxis genes

The top 30 list of up-regulated genes from the *spf* mutant expression profiling is enriched in genes involved in motility and chemotaxis [*cheV*, *flaA* (subunit E), *flaA* (subunit C), *flaD*, *flaF*, *flgB*, *flgF*, *flgM*, *fliC*, *motX*, VSAL_I0799 (methyl-accepting chemotaxis protein) and VSAL_I2193 (methyl-accepting chemotaxis protein)]. In total, 25 motility and chemotaxis genes were up-regulated in the *spf* deletion mutant. Out of these, 16 are located from gene 2315-2340 on chromosome I (VSAL_I2315-2340) in a region highly enriched in motility and chemotaxis genes (VSAL_I2283-2343). In a biological context (i.e., in the wild-type strain), these findings indicate a downregulation of chemotaxis and motility genes in the presence of glucose. The results make biological sense because the bacterium does not require to be motile or swim towards glucose during an infection inside a host. A similar change in expression of genes involved in motility has also been reported at different salt concentrations (Karlsen et al., 2008). Under seawater-like conditions, *A. salmonicida* is expressing elevated levels of flagellins and is highly motile, whereas the bacterium is non-motile and expresses significantly lower levels of flagellins under physiological-like salt conditions.

A total of 15 chemotaxis and motility genes in the *VSsrna24* deletion mutant are significantly down-regulated. These genes include 10 of which are encoded within

VSAL_I2317-2337. Interestingly, 12 of the down-regulated genes correspond to genes are that are up-regulated in the *spf* deletion mutant. In absence of glucose, the bacteria might use chemotaxis and motility genes in the search for a host. In *E. coli*, chemotaxis towards several sugars, including glucose, has been described (Adler et al., 1973; Adler and Epstein, 1974; Lengeler et al., 1981). Motility of *A. salmonicida* wild-type and the *spf, VSsrna24,* and *spf*/*VSsrna24* deletion strains was tested by growing the bacteria on semi-solid agar plates (LA plates with 0.25 percent agar) and subsequent monitoring of the swim zone diameters. Figure 8 shows that the wild-type and *spf* mutant strains swim at comparable rates, whereas the *VSsrna24* and *spf*/*VSsrna24* mutants are virtually unable to swim. This is in good agreement with the microarray results. The four different strains have comparable growth rate, and we therefore conclude that the differences in swim zone diameters are due to differences in motility and not growth per se.

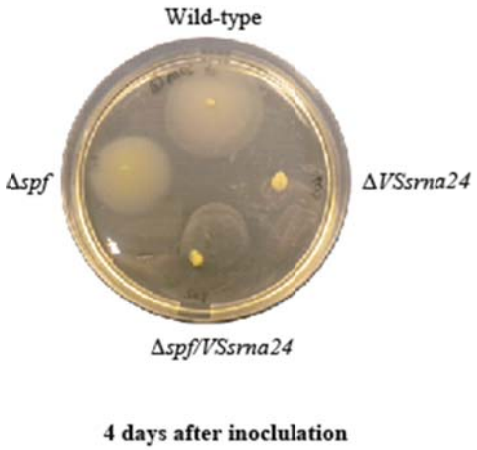

Figure 8. Wild-type and *spf, spf/VSsrna24,* and *VSsrna24* deletion strains plated onto semi-solid agar plates and incubated for four days.

In summary, a large number of motility and chemotaxis genes seem to be negatively and positively regulated by Spot 42 and VSsrna24, respectively. When compared to the expression pattern of the two sRNAs, these data indicate that Spot 42 down-regulates motility and chemotaxis genes in presence of glucose, while VSsrna24 up-regulates many of the same genes under conditions without glucose.

4. Concluding remarks

In its natural habitats, *A. salmonicida* is faced with environments of changing nutritional composition. To cope with these changing environments, the bacteria need to regulate their gene expression accordingly. For example, during an infection, the bacteria express another set of genes than outside the host. Inside a host, the availability of, e.g., iron and glucose differ compared to the outside. The bacteria are exposed to high iron concentrations during an infection, but the iron is bound to compounds inside the host and therefore not available. Results from **Paper 1** identified a number of genes differentially expressed under iron-restrictive conditions. By studies of the *A. salmonicida* Fur regulon, we were able to identify genes under control of Fur. Under iron-restrictive conditions, i.e., inside a host, the bacteria down-regulate iron-using genes and express sequestering systems to steal iron from the host. An sRNA identified in **Paper 2**, RyhB, is under control of Fur and plays a role in the post-transcriptional regulation of the Fur regulon.

sRNAs accumulate and play important roles in post-transcriptional gene regulation under specific (often stress) conditions (Vogel and Wagner, 2005). The whole-genome sRNA prediction in **Paper 2** focused on IGR-encoded sRNA genes expressed under different conditions and experimentally verified nine novel sRNAs. As reported in **Paper 3** and **Paper 4**, the expressions of the *A. salmonicida* Spot 42 homolog and novel VSsrna24, respectively, both respond to glucose. During an infection, the bacteria are exposed to high glucose concentrations. The Spot 42 and VSsrna24 expression patterns indicate that Spot 42 is expressed at high glucose concentration, i.e., during an infection, whereas VSsrna24 is expressed at low glucose concentrations, i.e., outside a host.

Results from **Paper 3** propose Spot 42 to down-regulate genes involved in uptake of other carbohydrates than glucose in a CCR-like manner and to repress genes involved in motility and chemotaxis. During an infection, the uptake systems for other carbohydrates than glucose are not required, and the bacteria do not require to be motile in order to swim towards glucose. In **Paper 4**, VSsrna24 is suggested to down-regulate uptake systems for glucose and other carbohydrates in conditions without glucose and to up-regulate genes involved in motility and chemotaxis.

In summary, Spot 42 and VSsrna24 are suggested to work in concert to regulate, directly or indirectly, a number of the same genes under different conditions. Under the high glucose conditions during an infection, Spot 42 is expressed to down-regulate uptake systems for other carbohydrates than glucose. Spot 42 also down-regulates genes required for motility and chemotaxis as the ability to swim is not required. VSsrna24 is expressed in conditions outside a host to down-regulate the PTS system in conditions of low carbohydrate concentrations in the environment. Outside a host, VSsrna24 up-regulates genes for motility and chemotaxis, which the bacteria utilize in their search for a carbon source.

To follow up the work from this project, a number of potential experiments to shed further light on selected topics are being planned or have been started. Firstly, an extended whole-genome sRNA prediction which spans the complete genome, not only the IGRs, might identify sRNA genes located antisense to, or within, protein coding regions. Such a study should preferably be complemented by an unbiased trancriptome analysis method, such as RNA-seq. To gain further insight into the *A. salmonicida* sRNA pool, RNase E and Hfq deletion mutants could be included in such studies. An *rne* (encoding RNase E) deletion mutant is hypothesised to result in accumulation of sRNAs that normally are degraded in an RNase E-dependent manner. Deleting *hfq* from the genome might produce a strain with reduced sRNA-mRNA stability. Secondly, predicted binding partners of the studied sRNAs Spot 42 and VSsrna24 should be verified experimentally. sRNA-mRNA binding can be validated by gel mobility shift assays of *in vitro* transcribed sRNAs and predicted binding partners in presence of Hfq, fusion-reporter assays, structure probing, and/or site directed mutagenesis. Finally, additional predicted and experimentally verified Fur binding sites should continuously be added to the vibrio alignment matrix to help create new and improved Fur box predictions.

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Paper 1

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Paper 2

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Paper 3

Geir Åsmund Hansen, Rafi Ahmad, Erik Hjerde, Christopher G. Fenton, Nils-Peder Willassen, Peik Haugen (2011). **Expression profiling of a *spf* deletion mutant suggests biological roles and mRNA targets for Spot 42 in the fish pathogen *Aliivibrio salmonicida*.** Submitted to *RNA Biology*.

Paper 4

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